Optimising the efficacy of plasmid DNA vaccines

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By

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Abstract

Plasmid DNA offers a novel mechanism to deliver antigens *in vivo*. Both humoral and cellular responses are induced in the systemic and mucosal compartments following mucosal administration. These properties suggest plasmid DNA represents a very promising technology for future vaccine development. However, the major disadvantage of this method of vaccination currently is the requirement for large doses of plasmid DNA needed to be efficacious in man. Therefore, by elucidating the mechanism underlying cellular transfection and improving the immunogenicity of plasmid DNA vaccines, the amount of plasmid DNA required to induce a protective immune response may be reduced. To further this goal, this study has investigated the use of cytofectins and translocation peptides to improve the efficiency of plasmid DNA delivery, and to enhance the immune response by co-delivery of genetic adjuvants.

The optimal parameters to complex cytofectins with plasmid DNA were compared in their efficiency to transfect cells *in vitro*. The efficiency of cytofectin complexed plasmid DNA delivery was also investigated *in vivo*, by administering plasmid DNA cytofectin complexes by the intranasal route. Dendritic cells containing the transgene were observed at the site of administration and at the draining lymph node.

This study also investigated a novel delivery system for plasmid DNA delivery. We have investigated the properties of the homeoprotein Antennapedia in complexing plasmid DNA and demonstrated enhanced transfection compared to naked plasmid DNA *in vitro*. Finally, this study investigated the chemokine CCL20 to act as a molecular adjuvant. CCL20 was cloned into an expression vector and after intranasal or intradermal delivery can increase the number of dendritic cells recruited to the site of administration.

In summary, this study has demonstrated that the efficacy of plasmid DNA can be improved through optimising delivery and that by using molecular adjuvant, CCL20, the number of antigen presenting cells present at the site of vaccine delivery can be increased.

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Abbreviations

APC – Antigen presenting cell

BALT – Bronchial Associated Lymphoid Tissue

BMDCs – Bone marrow derived DCs

CD – Cluster of Differentiation

cDNA – Complimentary Deoxyribonucleic Acid

CMV – Cytomegalovirus

CTL – Cytotoxic Lymphocyte

DCs – Dendritic Cells

DNA – Deoxyribonucleic

FCS – Foetal Calf Sera

Flt3L – fms like tyrosine kinase 3 Ligand

GFP – Green Fluorescent Protein

GM-CSF – Granulocyte/Macrophage Colony Stimulating Factor

IFN – Interferon

IL – interleukin

Lux – Luciferase

mAb – Monoclonal Antibody

MALT – Mucosal Associated Lymphoid Tissue

MHC – Major Histocompatability Complex

mRNA – messenger RNA

NALT – Nasal Associated Lymphoid Tissue

PLG – Poly-lactide-coglycolide

PP – Peyer's Patch

RANTES – Regulated upon activation, Normal T cell expressed and Secreted)

RNA – Ribonucleic Acid

SDS - Sodium Dodecyl Sulphate

SEAP – Secretory Alkaline Phosphatase

Th1/2 – Helper T lymphocytes 1/2

TNF – Tumour Necrosis Factor

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Chapter 1 – Literature Review

1.1 Vaccination

In 1796 Edward Jenner demonstrated that using liquid from cow pox sores, he could protect against small pox infection. Thus coining the term "vaccination" from the latin word for cowpox, vaccinia. However, it was in 1880 when Louis Pasteur further contributed to the study of vaccines when he reported to the French Academy of Science that "…chicken cholera is produced by a microscopic parasite, that there exists an attenuated virus of that disease, and that one or more inoculations of this attenuated virus can preserve the animals from the moral effects of a later inoculation….".

As a result of whole spread vaccination, endemic childhood infectious diseases have virtually disappeared from industrialised countries. These are remarkable successes, which together with clean water and antibiotics have profoundly affected human society (Bland and Clements 1998). However, there are still many infectious diseases that do not currently have a working vaccine, for example Malaria. Therefore, we need to utilise modern scientific knowledge to design efficacious vaccines.

1.1.1 Designing Vaccines

Several factors must be kept in mind in developing a successful vaccine. First and foremost, the development of an immune response does not necessarily mean that a state of immunity has been achieved. Often the quality of the immune response is critical, for example biasing a response towards the Th1 pathway, may be protective for some organisms, but in others could exacerbate the disease. A second factor is the development

of immunological memory. For example a vaccine that induces a protective primary response may fail to induce memory-cell formation, leaving the host unprotected after the primary response to the vaccine subsides. This increases the number of vaccinations required, reducing patient compliance and the proportion of the herd protected.

1.1.2 Classic methods of Vaccine Development

Since the time of Pasteur until recently there have been 2 mains paths of vaccine development: attenuation and inactivation. Attenuated vaccines are organisms that have are viable but are non-pathogenic. Whereas, inactivated vaccines are either parts of organisms (subunit vaccines) or organisms that have been killed (Inactivated vaccines).

1.1.2.1 Attenuation

The first attenuated vaccines were created by Pasteur for rabies and anthrax. This was accomplished through barraging the organism using heat, oxygenation, chemical agents or aging. Passage in animal hosts, such as embryonated hen's egg was practiced by Theiler for a yellow fever vaccine, and then with the development of cell culture in the 1940's, attenuation *in vitro* was accomplished by a variety of means including selection of chance mutants, adaptation to growth at low temperatures, chemical mutation to induce inability to grow at high temperature (temperature sensitivity), or induction of auxotrophy in bacteria.

1.1.2.1a Poliomyelitis

An example of a successful vaccine is the polio vaccine, with the wild polioviruses being limited to only 2 parts of the world – sub-Saharan Africa and the Indian subcontinent. However, the virus will never be totally eradicated as the vaccine itself acts a repository. Although the attenuated poliovirus strains were passaged more than 70 times in animals or cell culture, the property of attenuation rests on a small number of mutations (Martin and Minor 2002). Those mutations change only 1% of amino acids, and most of the changes have nothing to do with attenuation. Moreover, after oral administration to humans, reversion of the few attenuating mutations is a constant feature of replication in the intestine, where neurovirulent viruses have a selective advantage.

The other facet of the problem is that recombination between poliovirus serotypes and between poliovirus and enteroviruses is a regular phenomenon during replication in the intestine. When the 5' end of a reverted poliovirus combines with the 3' end of another enterovirus, the result is a virus that not only is neurovirulent but also can be transmitted easily and become epidemic (Kew *et al.* 2002). Epidemic vaccine-derived polio has happened in the past in Egypt, China, Israel, the Dominican Republic, Haiti, the Philippines and Madagascar, undoubtedly accompanied by hundreds of asymptomatic infections. In addition, rarely persons with B cell deficiencies chronically excrete reverted vaccine virus, for periods of greater than 10 years (Kew *et al.* 1998; Cherkasova *et al.* 2002).

1.1.2.2 Inactivated organism or subunit

Late in the 19th century, Theobald Smith in the United States and Pasteur`s colleagues independently showed that whole organisms could be killed without losing immunogenicity, which soon became the basis of vaccines for typhoid and cholera and later for pertussis, influenza and hepatitis A. In the 1920s, the exotoxins of *Corynebacterium diphtheriae* and *Clostridium tetani* were inactivated by formalin to provide antigens for immunisation against diphtheria and tetanus. Later in the 20th century influenza vaccine progressed to subunit preparations, and pertussis vaccine progressed from bacterial soup to the extracted proteins that we use today in acellular vaccines. Extracted native polysaccharides from the capsules of *Haemophilus influenzae* type b, pneumococci, meningococci, and typhoid bacilli proved useful in immunising older children and adults, and more recently, the conjugation of these polysaccharides with proteins have provided us with immunogens that generate T cell memory and are effective even in young infants.

Although these vaccine strategies have been successful, they have not been able to address some of the most deadly diseases. Effective vaccines are still required for HIV, Tuberculosis, and Malaria. Novel strategies to induce an immune response and act as a vaccine vector have been investigated. These include the potential to use plasmid DNA to induce an immune response against an encoded antigen.

1.1.3 Vaccine Strategies

1.1.3.1 Recombinant Vector Vaccines

It is possible to introduce genes encoding major antigens of especially virulent pathogens into attenuated viruses or bacteria. The attenuated organism serves as a vector, replicating within the host and expressing the gene product of the pathogen. A number of organisms have been investigated as potential vector vaccines, including vaccinia virus, the canarypox virus, attenuated poliovirus, adenoviruses, attenuated strains of *Salmonella*, and the BCG strain of *Mycobacterium bovis*.

The viral vectors recently in laboratory and clinical use are based on RNA and DNA viruses possessing very different genomic structures and host ranges. Particular viruses have been selected as gene delivery vehicles because of their capacities to carry foreign genes and their ability to efficiently deliver these genes associated with efficient gene expression. These are the major reasons why viral vectors derived from retroviruses, adenovirus, adeno-associated virus, herpesvirus and poxvirus are employed in more than 70% of clinical gene therapy trials worldwide. Among these vector systems, retrovirus vectors represent the most prominent delivery system, since these vectors have high gene transfer efficiency and mediate high expression of gene products. Members of the DNA virus family such as adenovirus-, adeno-associated virus or herpesvirus have also become attractive for efficient gene delivery as reflected by the fast growing number of clinical trials using these vectors. The first clinical trials were designed to test the feasibility and safety of viral vectors. Numerous viral vector systems have been developed for ex vivo and in vivo applications. More recently, increasing efforts have been made to improve

infectivity, viral targeting, cell type specific expression and the duration of expression. These features are essential for higher efficacy and safety of RNA- and DNA-virus vectors. From the initial investigations that utilised viral vectors it was apparent that they harbour risks such as toxicities, immunoresponses towards viral antigens or potential viral recombination that limited their clinical use. However, many achievements have been made in vector safety, the retargeting of virus vectors and improving the expression properties by refining vector design and virus production.

One potential of recombinant viral vectors that has been successful in the literature is a prime boost strategy. Priming is accomplished with a plasmid DNA encoding the antigen of choice, and is followed by boosting with a viral vector (Pinto *et al.* 2003). Thereby increasing the magnitude and duration of the response as compared to boosting with plasmid DNA alone. Studies have demonstrated that previous immunological exposure to modified Vaccinia-Ankara virus (MVA) reduced the efficiency of subsequent priming and boosting using the same vaccine vehicle. However, a combined regime whereby the animals were first primed with the DNA vaccine and then boosted with MVA was the most potent protocol for the induction of both interferon-gamma-producing and cytolytic T cells against two CTL epitopes simultaneously (Hanke *et al.* 1998).

1.1.3.2 Synthetic Peptide Vaccines

Among the synthetic peptide vaccines currently being evaluated are those for hepatitis B virus, the malaria parasite, diphtheria toxin, influenza and HIV. Construction of synthetic peptides for use as vaccines to induce either humoral or cell-mediated immunity requires knowledge of the T-cell and B-cell epitopes of the antigen. In the design of synthetic

peptides, the primary structure of the antigen is analysed to identify strongly hydrophilic sequences, which most likely correspond to B-cell epitopes. Ideally immunodominant B-cell epitopes are identified for use as a synthetic peptide vaccine. However, a successful vaccine also requires a cell-mediated immune response; therefore immunodominant T helper cell epitopes are required. These peptides must have a site (the agretope) that enables them to interact with the MHC molecules as well as a site (the epitope) that enables them to interact with the T cell receptor. Since MHC molecules differ in their ability to present peptides to T cells, MHC polymorphism within a species influences the level of T-cell responsiveness by different individuals to different peptides.

1.1.3.4 Multivalent Subunit Vaccines

The limitation with synthetic peptide vaccines and recombinant protein vaccines is that these vaccines tend to be poorly immunogenic; in addition, they tend to induce a humoral antibody response but are less likely to induce a cell-mediated response. Therefore, a vaccine needs to contain both immunodominant B and T cell epitopes. If a CTL response is desired the vaccine must be delivered intracellularly so that the peptides can be processed and presented together with class I MHC molecules.

One approach is to prepare solid matrix-antibody-antigen (SMAA) complexes by attaching monoclonal antibodies to particulate solid matrices and then saturating the antibody with the desired antigen. By attaching different monoclonal antibodies to the solid matrix, it is possible to bind a mixture of peptides or proteins, composing immunodominant epitopes for both T cells and B cells, to the solid matrix (Alber *et al.* 2000). These multivalent complexes have been shown to induce vigorous humoral and

cell-mediated responses. Their particulate nature contributes to their increased immunogenicity by facilitating phagocytosis.

1.1.3.5 Anti-Idiotype vaccines

Monoclonal anti-idiotypic antibodies (ab2 MAbs), which bear internal images of neutralization epitopes of viruses, are potentially useful as antiviral vaccines. Such ab2, MAbs evoke virus neutralizing anti-anti-idiotypic (ab3) antibodies that might protect immunized animals against virulent viral disease. Anti-idiotype vaccines have been shown to induce protective immunity in mice against hepatitis B virus and several other pathogens (Zanetti *et al.* 1987). The development of an anti-idiotype vaccine for humans hold much promise when immunisation with a killed or attenuated vaccine would pose an unacceptable risk.

1.2 DNA Vaccines

1.2.1 Historical background.

In 1960 the first use of a naked DNA was documented by Ito, who demonstrated the induction of papillomas in rabbit skin by injection of purified nucleic acid extracted from the Shope rabbit papilloma virus. However, it was not until 1990 that the true potential of this method of vaccination was visualised. Wolff et al reported that intramuscular injection of plasmid DNA in a simple saline solution could transfect muscle cells *in vivo* and persist in an episomal form, thus potentially able to induce an immune response against the expressed proteins. In 1992, Tang et al were the first to show the induction of antigen specific antibodies resulting from plasmid DNA injection via a "gene gun" into mice However it was in 1993, when Ulmer et al demonstrated that mice could be

protected from influenza virus challenge by the induction of antibody and CD8 cytotoxic T lymphocytes (CTL) responses when previously immunised with a DNA vaccine, that the true potential of this method of vaccination came to fruition. Since then, the immunogenicity or protective efficacy of DNA vaccines in preclinical models of infectious disease, allergy and cancer has been established in several animal species and attests to the simplicity and robustness of the technology.

1.2.2 Basic elements of a plasmid vector

1.2.2.1 Description of the plasmid vector

The basic elements of a bacterial vector used as a DNA vaccine are: a promoter and enhancer (to enable transcription of the inserted foreign gene); an mRNA transcription termination/polyadenylation signal (for directing expression in mammalian cells); an antibiotic resistance gene (to confer an antibiotic selectable marker for growth in E.Coli.); an origin of replication for growth in E. Coli; and specific nucleotide sequences, cytidine-phosphate-guanosine (referred to as CpG motifs) which are stimulatory for lymphocytes and act as an adjuvant.

1.2.2.2 Promoter/enhancer region within a plasmid vector

There are 2 main features within the bacterial plasmid DNA vector that are essential for optimal expression within mammalian cells. These are a strong promoter/enhancer and the transcription/termination sequence. The majority of DNA vaccines contain either the human cytomegalovirus (CMV) immediate/early (IE) promoter (Boshart *et al.* 1985). the

SV40 early promoter (Moreau *et al.* 1981) or the Rous Sarcoma virus LTR (Gorman *et al.* 1982). In conjunction with one of these promoters, a polyadenylation sequence from either bovine growth hormone (BGH) or SV40 is required to stabilise the mRNA (Pfarr *et al.* 1986). A caveat to the use of these promoters are the reports that interferon-γ (IFN-γ) is released in situ during the priming of an immune response (as a result of the inflammatory cytokine inducing properties of bacterial DNA), thus suppressing transcription from many viral promoters (Harms and Splitter 1995; Romero and Lavine 1996). This in turn can affect the magnitude of the immune response. by limiting antigen production for B and T cell priming. There have been studies using alternatives to virus derived control elements for example promoter/enhancer sequence driving expression of MHC class I and II (Xiang *et al.* 1997; Gebhard *et al.* 2000), dectin in Langerhan' cells (Bonkobara *et al.* 2001) and muscle specific genes (Barnhart *et al.* 1998; Kwissa *et al.* 2000) have all been investigated, however the levels of expression do not exceed those generated from the HCMV IE promoter combined with the CMV intron A. Further optimisation of expression has been reported by the inclusion of a 'Kozak' translational initiation sequence 5' to the start codon of the inserted 'foreign' gene.

1.2.3 Cellular interaction of plasmid DNA

The precise mechanism by which naked plasmid DNA enters cells and is translocated to the nucleus to initiate transcription is not fully understood. Evidence from electron micrographs suggests plasmid DNA is internalised by electrostatic binding to the cell membrane and endocytosed via clathrin coated pits (Zabner *et al.* 1995; Friend *et al.* 1996). Direct fusion with the cell membrane and/or fluid phase endocytosis

(macropinocytosis) (Apodaca 2001) may also contribute to cellular uptake of plasmid DNA. Once plasmid DNA is internalised, rapid escape from the endosome is critical to the efficiency of plasmid encoded antigen expression. Endosomes mature through various stages of acidification and eventually fuse with lysosomes containing enzymes, which degrade DNA. Release of plasmid DNA from the endo-lysosome is thought to be a consequence of membrane disruption. Cationic lipids or peptides (eg from the haemagglutinin of influenza virus) which fuse with the endosomal membrane have been reported to increase transfection efficiency (Fasbender *et al.* 1997; Zhang *et al.* 2001). Despite these modifications only a fraction of internalised plasmid DNA penetrates the cytoplasm. Plasmid DNA is prone to further degradation by cytosolic nucleases, thus only a very small proportion of transfected DNA is available for transport across the nuclear membrane (Johnson-Saliba and Jans 2001). The size of plasmid DNA vectors is generally assumed to preclude efficient diffusion across the nuclear pore complex, thus compounding the inefficiency of plasmid DNA transfection. Following transcription, mRNA is translated and plasmid-encoded protein within the cytoplasm is accessible to the TAP dependant MHC class I processing pathway.

1.2.4 Generation of Immune Responses by DNA Vaccines

1.2.4.1 Processing and presentation of plasmid DNA

For the presentation of plasmid DNA encoded antigen to B cells, it appears that as long as antigen is accessible to the B cell receptor, the nature of the transfected cell exporting the plasmid DNA encoded protein is immaterial (Loirat *et al.* 1999; Armand *et al.* 2000).

For DNA vaccines expressing purely cytosolic proteins it had been suggested that antigen release to the extracellular milieu might be affected by the CTL lysis of the transfected cell. However, recent data indicated that neither perforin nor CD8+ T cell mediated lysis is required for priming CD4+ T cell or antibody responses (Hassett *et al.* 1999)

For the priming of CD8+ T cell responses there are at least three mechanisms by which plasmid DNA encoded antigen may be processed and presented. These include (i) direct transfection and priming by somatic cells (such as myocytes, fibroblasts and kerationcytes), (ii) cross priming, whereby antigen is transferred from transfected somatic cells to professional APCs or (iii) direct transfection of APCs.

1.2.4.2 Direct priming

The observation that intramuscular injection of plasmid DNA induced protein expression in muscle (Wolff *et al.* 1990) and elicited strong CD8+ CTL responses in mice (Ulmer *et al.* 1993) suggested that muscle cells were critically involved in the initiation of CTL responses. In support of this, was shown that adoptive transfer of stably transfected myoblasts expressing influenza nucleoprotein into mice was sufficient to induce CD8+ CTL and protect from virus challenge (Ulmer *et al.* 1996). This showed that antigen expression by muscle cells was sufficient to prime for CTL-mediated protection. Unresolved was whether CTL responses were directly induced by myocytes expressing the encoded antigen or were indirectly induced through antigen transfer from myocytes to professional APCs. To resolve this issue, a study examining somatic versus bone marrow derived APC CTL priming was undertaken. Bone-marrow derived cells were adoptively

transferred to irradiated F1 hybrid mice, to create bone marrow chimeras with somatic cells of both F1 MHC haplotypes and APCs of a single MHC haplotype of the donor bone marrow. Peptide-specific CTL were only induced by immunisation of mice with plasmid DNA encoding the peptide presented by the MHC class I molecules found on the donor bone marrow (Fu *et al.* 1997; Iwasaki *et al.* 1997). These studies demonstrated that bone marrow derived APC play a dominant role in CTL priming and suggested that either APCs were directly transfected or that antigens (or their epitopes) synthesized in somatic cells could be transferred to APCs for presentation by MHC class I molecules.

1.2.4.3 Cross-priming by DNA vaccines.

Cross-presentation of antigenic material from apoptotic cells to DCs has been established as an important mechanism for the induction of CD8+ CTL responses against tumour or pathogen derived antigens (Bevan 1976). *In vitro* studies have established that this mechanism is distinct from the classical endogenous TAP dependent pathway of MHC class I restricted peptide presentation. Several lines of evidence indicate that cross-priming also occurs following DNA vaccination. Loriat et al, 1999, have shown that vaccination with a plasmid DNA vector driven by a muscle specific promoter elicited CTL despite expression of antigen restricted to muscle cells, suggesting that cross presentation of antigen to APC was involved. Second, Corr et al, 1999, demonstrated using a transactivating plasmid system and bone marrow chimeras that CTL induction by plasmid DNA was critically dependant upon antigen expression by somatic cells and transfer to APCs. However, a complementary mechanism involving direct transfection of APCs could not be excluded. More recently, cross-presentation of antigen to DCs has

been facilitated by constructing DNA vaccines that induce apoptotic death of the antigen-expressing cell. Co-immunisation with DNA vaccines which express Fas or a mutated version of a caspase gene which induce the creation of apoptotic bodies has been reported to enhance cell mediated immunity (Chattergoon *et al.* 2000; Sasaki *et al.* 2001). The underlying mechanism by which antigen is transferred from somatic cells to APCs is not fully understood. Chaperones, including heat shock protein 70 and 73 (hsp70, hsp73) have been implicated in the transfer of immunogenic material from plasmid DNA transfected macrophages or somatic cells to DCs (Kumaraguru *et al.* 2000; Kammerer *et al.* 2002). The precise mechanism by which chaperone-bound peptides are released and cross-presented by DC's remains to be resolved. Candidate mechanisms include either a facilitated exchange or delivery system, whereby chaperone-bound peptide is internalised following receptor mediated DC binding with peptide exchange between nascent MHC class I molecules and the chaperone. Alternatively chaperone-bound peptide may be directly exchanged with cell surface MHC class I bound peptide.

1.2.4.4 Direct transfection of antigen presenting cells

While there is evidence for cross-priming, other studies have provided evidence that APCs are directly transfected following DNA vaccination. Removal of muscle (within 10 minutes) from the injection site of a DNA vaccine did not alter the magnitude or quality of the subsequent immune response, suggesting transfection of cells (possibly APCs) distal to the injection site is sufficient to prime CTL (Torres *et al.* 1997). Antigen and reporter genes have been demonstrated in macrophages, Langerhan' cells and DCs present both at the site of DNA vaccine delivery and in draining lymph nodes

(Chattergoon *et al.* 1998; Akbari *et al.* 1999; Barnfield *et al.* 2000). This suggests that APCs are transfected *in situ* and migrate to draining lymph nodes or alternatively are transfected within lymph nodes following lymphatic drainage of plasmid DNA. Conclusively, plasmid encoded mRNA has been demonstrated in isolated DCs following either intradermal or mucosal vaccine delivery (Bouloc *et al.* 1999; Barnfield *et al.* 2000). Functional studies have demonstrated that DCs isolated from plasmid DNA injected skin or nasal tissue were able to stimulate the proliferation of T cell responses in naïve mice following adoptive transfer (Bouloc *et al.* 1999). These reports indicate that different routes of DNA vaccine delivery result in direct transfection of APCs and lead to induction of CTL responses. Current evidence suggests the efficiency of DC transfections *in vivo* is very low (Barnfield *et al.* 2000). However, considering the potent stimulatory activity of DCs (Steinman and Pope 2002), large numbers of transfected DC may not actually be essential to elicit CTL responses. In support of this hypothesis, adoptive transfer of as few as 500-1000 *in vitro* transfected DCs to mice induced a comparable cellular and humoral immune response as that elicited by gene gun immunisation with the same antigen encoding plasmid (Timares *et al.* 1998).

1.2.5 Augmenting the Immunogenicity of DNA Vaccines

Pre-clinical studies, in several murine models of infectious disease have shown that DNA vaccines effectively elicit cellular and humoral immune responses, which confer protection from pathogen challenge (Ulmer *et al.* 1993; Gebhard *et al.* 2000). Whilst these observations have been very encouraging, the magnitude of the immune responses induced in human and non-human primates by the first generation of naked DNA

vaccines has proven lower than that achieved by attenuated or protein adjuvanted vaccines (MacGregor *et al.* 1998; Amara *et al.* 2001). This difference in efficacy of DNA vaccines between species is in part due to the higher level of protein expression achieved in mice following intramuscular injection than in other species (Jiao *et al.* 1992). Secondly, the dose of plasmid DNA injected in mice per Kg body weight, significantly exceeds the per Kg dose administered to human and non-human primates, thus comparisons of immunogenicity are not equal. Furthermore, murine cells reportedly demonstrate greater sensitivity than human cells to the adjuvant activity of CpG DNA (Bauer *et al.* 2001). Thus while naked DNA vaccines on their own may not be adequate to protect against disease in man, technological developments in vector construction, incorporation of genetic or conventional adjuvants, deployment of novel delivery vehicles or poxvirus booster inoculations may lead to the development of effective DNA vaccines.

1.2.5.1 Modification of the Vector

It is generally believed that the level of gene expression attained *in vivo* following DNA vaccination correlates with the immune response generated. Therefore, approaches to improve gene expression may enhance DNA vaccine potency. Virally derived promoters, including the most frequently used CMV immediate early enhancer-promoter provide high levels of gene expression within mammalian cells (Manthorpe *et al.* 1993). However, these promoters are subject to transcriptional silencing, following CpG DNA mediated activation of proinflammatory cytokines (Romero and Lavine 1996). Elimination of CpG motifs within the plasmid backbone reportedly increases the duration

of antigen expression (Yew *et al.* 2001). Whilst this modification may provide the longer term expression required for gene replacement therapy, this needs to be balanced with the loss of adjuvant activity required for DC maturation and to initiate T cell priming. Alternative promoters, including cell-specific, tissue specific and hybrid CMV promoters have been evaluated (Xiang *et al.* 1997; Barnhart *et al.* 1998; Gebhard *et al.* 2000; Kwissa *et al.* 2000; Bonkobara *et al.* 2001). Plasmid vectors containing the human ubiquitin promoter could be particularly useful. Sustained, high level gene expression has been achieved with this promoter in the presence of plasmid backbones containing CpG motifs (Yew *et al.* 2001).

1.2.5.2 Codon optimisation

Optimising codon usage for eukaryotic cells can also enhance expression of antigens. Codon bias has been observed in several species and may differ from the codons most frequently used by pathogens. Changes in codon usage, which modify RNA structure, have been shown to enhance the expression of HIV *env* and *gag* genes. In mammalian cells the *env* and *gag* genes are made as long unspliced transcripts that contain overlapping reading frames with the small regulatory genes of HIV *rev* and *tat*. These transcripts are retained in the nucleus and are rapidly spliced in the absence of rev, thus preventing the transport of full-length message to the ER. The transcripts are inhibited from transport to the ER by rev dependent sequences that are themselves part of the structural gene sequence. Structural modification of the RNA through changing codon usage has led to an increase in HIV envelope and gag expression with an increase in HIV gp160 CTL and antibody reported (Vinner *et al.* 1999; Andre *et al.* 2000).

1.2.5.4 CpG motifs of plasmid DNA

Bacterial plasmid DNA contains immunostimulatory sequences (ISS) consisting of cytidine-phosphate-guanosine (CpG) dinulceotide sequences, usually flanked by two 5' purine and two 3' pyrimidines (Sato *et al.* 1996). Stimulatory bacterial CpG sequences (CpG-S) differ from vertebrate DNA, in that they are unmethylated and expressed at the expected frequency (predicted from random base utilisation) whereas this frequency is suppressed 20-fold in vertebrate genomes (Bird 1986). These structural differences are recognised as "foreign" by vertebrate immune systems and directly activate the innate immune system to produce multiple proinflammatory cytokines, including TNF-α, IL-6, IL-12, IL-18 and IFN-α and IFN-γ (Klinman *et al.* 1996) which enhance the ability of the antigen presenting cells (APC) to present antigen and stimulate T cell activation. When the ampR gene was substituted for a kanR selectable marker, which contained additional CpG motifs, the modified plasmid was shown to induce an enhanced CTL and IgG response compared to the original vector (Sato *et al.* 1996). However, this ability of CpG to stimulate an immune response is abrogated by *in vitro* methylation (Pasquini *et al.* 1999). Co-administration of an empty vector (i.e. without any antigen-coding sequence) may boost the immune response to a DNA vaccine; however there appears to be an upper limit to the ability of additional CpG motifs to improve the immune response (Krieg *et al.* 1998).

1.2.5.5 Immunostimulatory effects and mechanisms of CpG

Recognition of bacterial CpG is mediated by a conserved receptor, Toll like Receptor 9 (TLR) expressed by macrophages and DC's (Bauer *et al.* 2001). Current data suggests that CpG oligonucleotides cross the cell membrane via sequence non-specific-receptor mediated endocytosis and are then recruited to an endosomal compartment where TLR9 co-localises (Ahmad-Nejad *et al.* 2002). This is possibly due to CpG release from upon plasmid DNA degradation by extracellular nucleases. Endosomal acidification of CpG DNA is required to initiate signalling via the Toll/IL-1R pathway, since specific inhibitors such as cholorquine block the immunostimulatory activity of CpG DNA (Hacker *et al.* 1998). In macrophages and DCs, CpG-DNA mediated TLR9 signalling is transduced by a common adapter protein, myeloid differentiation factor 88 (MYD88) which results in the down stream generation of intracellular reactive oxygen species (Yi *et al.* 1998), activation of the mitogen activated kinases (MAPK) and nuclear factor κB (NFκB), (Yi and Krieg 1998; Yi and Krieg 1998). The biological outcome of CpG driven TLR9 ligation in DC's is secretion of T helper 1 (Th1) polarising cytokines (IL-12 and IL-18), upregulation of co-stimulatory molecules (CD40, Cd80 and CD86) and functional maturation for antigen presentation (Sparwasser *et al.* 1998; Schattenberg *et al.* 2000). These observations indicate that the plasmid backbone functions as a Th1 polarising adjuvant in DNA vaccines and explains the IgG2a antibody and CTL responses seen following intramuscular immunisation. CpG also delivers T-independent survival signals to DCs that are mediated by up-regulation of the cellular inhibitor of active caspase (cIAPs), (Park *et al.* 2002). This might be a mechanism by which CpG DNA may prolong DC presentation of naked DNA encoded antigen and compensate for the relative low

levels of antigen produced (compared to replicating vectors). The signalling pathways activated by CpG DNA in B cells stimulate the secretion of IL-6, IL-10 and IL-12 and also immunoglobulin in a polyclonal T-independent manner (Krieg *et al.* 1995). These CpG mediated effects synergise with signalling through the B-cell antigen receptor by antigen secreted *in situ* from naked DNA.

Immune recognition of CpG DNA varies between species, with mouse cells expressing a high-responder phenotype and human cells a low-responder phenotype (Bauer *et al.* 2001). The CpG motifs optimally active in mice are poor stimulators of innate immunity in human PBMC (Bauer *et al.* 2001). Extensive screening assays have identified structurally distinct CpG oligodeoxynucleotide (ODN) sequences, with enhanced stimulation for human cells. K-type CpG ODN stimulate IL-6 production by monocytes/DCs and B cell proliferation and IgM production. Whereas D-type CpG ODN preferentially stimulates IFN-γ production by NK cells (Verthelyi *et al.* 2001). Thus it may be possible to improve DNA vaccines for human use by the mutagenesis of endogenous plasmid CpG sequences. However, a relative increase in the levels of IFN-γ induced by CpG-DNA will be compensated by IFN-γ mediated suppression of viral promoter activity which drives antigen expression. A further level of complexity that is unravelling is the differential responsiveness of human DC subsets. Human plasmacytoid DC ($CD11c^+$ $CD123^+$) are responsive to CpG DNA, but monocyte derived $CD11^+$ DC lack TLR9 and do not appear to be stimulated (Bauer *et al.* 2001). Currently this difference is difficult to rationalise, in view of the front line defensive location of $CD11c^+$ DC at the body surfaces.

1.2.6 Genetic Adjuvants for DNA vaccines

1.2.6.1 Cytokines and Chemokines

Several groups have used plasmid DNA encoding various cytokine, chemokine or co-stimulatory molecules to enhance or polarise the type of immune response generated by DNA vaccination. In some instances the outcome attributed to a particular immunomodulator has been inconclusive or variable depending upon the antigen, mouse strain or plasmid vector used. The variability in response may also reflect the complexity of effects attributed to an immunomodulator and those elicited in response to bacterial components of the vaccine vector. Co-immunisation with plasmid DNA encoding Th1 cytokines, IL-12, IL-18, IFN-γ, and TNF-α or chemokines GM-CSF or RANTES with antigen coding DNA vaccine constructs has resulted in enhanced CTL and or Th-cell proliferative responses. Conversely, co-immunisation with plasmid DNA encoding certain Th2 cytokines (eg IL-4, IL-5, IL-6) or IL-12, IL-15, Il-18 or GM-CSF have been reported to augment humoral immune responses.

1.2.6.2 Co-stimulatory molecules

Other investigators have targeted transgene products to lymph nodes or APCs by constructing DNA vaccines encoding secreted fusion proteins containing CTLA-4, L-selectin or CD40L, whose cognate ligands are present on APCs. By targeting secreted antigens for APC uptake, stronger immune responses have been reported (Boyle *et al.* 1998; Sin *et al.* 2000). Similarly, expression of plasmid DNA encoded co-stimulatory

molecules (CD80 or CD86) on transfected somatic or APCs can provide critical secondary signals for T cell activation, through ligation of cognate receptors (CD28/CTLA-4). A number of studies have reported that co-administration of plasmid DNA encoding antigen and CD80 or CD86, increased T cell responses without any change in the humoral immune responses (Iwasaki *et al.* 1997; Kim *et al.* 1997).

1.2.6.3 Apoptotic molecules.

Studies have been undertaken to examine the effect of co-immunisation with an apoptotic inducing molecule, for example FAS. The *in vivo* killing of antigen bearing cells *in vivo* directly leads to the increased acquisition of antigen by APCs. This results in an increase of antigen specific CTL and the elaboration of T helper-1 (Th1) type cytokines and chemokines (Chattergoon *et al.* 2000).

1.2.7 DNA delivery

1.2.7.1 Electroporation

The distribution of plasmid DNA from the site of vaccine delivery and efficiency of cellular uptake are the key factors limiting the potency of DNA vaccines. One approach to increase DNA delivery is *in vivo* electroporation. A low intensity of electric pulses (100-200V/cm) delivered from electrodes applied to the muscle or skin has resulted in significant enhancement in the cellular and humoral immune responses generated to injected DNA vaccines (Kadowaki *et al.* 2000; Widera *et al.* 2000). The wide scale feasibility, user friendliness and safety of this vaccination approach remains to be established. The potential for DNA integration into host genomes is a concern, since

electroporation results in a much larger copy number of plasmid DNA per cell than that achieved by injected naked plasmid DNA (Dupuis *et al.* 2000).

1.2.7.2 Gene Gun

Gene gun technology uses a gas-driven biolistic bombardment device that propels particles coated with plasmid DNA directly into the skin (Williams *et al.* 1991; Tang *et al.* 1992; Fynan *et al.* 1993). These gold particles are propelled directly into the cytosol of target cells, resulting in transgene expression levels higher than those obtained by comparable doses of naked DNA and capable of inducing a protective immune response.

1.2.7.3 Liposomes

Liposomes are bilayered membranes consisting of amphipathic molecules (polar and nonpolar portions) such as phospholipids, forming unilayered or multilayered (lamellar) vesicles. Unilamellar vesicles have a single bilayer membrane surrounding an aqueous core and are characterised by either being small or large unilamellar vesicles, whereas multilayered vesicles have several lipid bilayers separated by a thin aqueous phase. Because liposomes can be prepared with significant structural versatility based on vesicle surface charge, size, lipid content, and co-entrapment of adjuvants, they offer considerable flexibility toward vaccine optimisation. The full scope of the use of liposomes to increase the effect of DNA vaccines is currently an active area of investigation. Intramuscular injection of plasmid DNA (hepatitis B surface antigen) entrapped in liposomes elicited 100-fold increased antibody titres and increased level of

cytokines when compared with those animals injected with naked DNA (Gregoriadis *et al.* 1997). A similar result on antibody augmentation was seen when DNA/liposome complexes were administered intranasally (Klavinskis *et al.* 1999).

1.2.7.4 Microparticle encapsulation

Another potential means of DNA delivery is the use of biodegradable polymeric microparticles. Plasmid DNA encapsulated by the polymer poly (lactide-co-glycolide, PLG) can be delivered by systemic and mucosal routes and has been reported to elicit immune responses (Chen *et al.* 1998; Kaneko *et al.* 2000) which confer protection in a murine rotavirus challenge model (Chen *et al.* 1998). Adsorption of plasmid DNA onto pre-formed PLG microparticles has also been shown to prime for both antibody and cellular immune responses (Denis-Mize *et al.* 2000). PLG mediated vaccine delivery appears to act in part, by targeting PLG containing microspheres to APCs rather than achieving an increase in gene expression (Denis-Mize *et al.* 2000).

1.2.8 Safety Issues

To date DNA vaccines have been shown to be well tolerated in both pre-clinical and phase 1 clinical trials. Immunopathological reactions such as inflammation and general immunosuppression have not been observed, although a number of safety concerns remain. These include (i) possibility that DNA vaccines could integrate into the host genome, which could result in the activation of oncogenes or the inactivation of tumour suppressor genes, (ii) induce responses against self antigens expressed on transfected

cells and initiate autoimmune disease, (iii) induce tolerance rather than active immunity to plasmid DNA encoded antigens and (iv) stimulate the production of antibodies to the injected DNA.

1.2.8.1 Potential for Integration

The Primary safety concern of plasmid DNA vaccines is their potential to integrate into the genome of host cells. This could increase the risk of malignancy by either causing the activation of oncogenes or inactivation of tumour suppressor genes. If integration occurs in germ line cells, there is also a potential for germline transmission. These concerns stem from the knowledge that plasmid can integrate at a very low level into cellular DNA when transfected into actively dividing cells. Though, it is believed that only a small fraction of integration events would alter a gene and prove harmful to a host cell. The probability of integration *in vivo* is likely to be further reduced by physical-chemical barriers, which impeded efficient uptake of plasmid DNA *in vivo*, including exonucleases, interstitial DNA-binding proteins and a lower rate of cellular proliferation from cells *in vitro* synchronised for transfection under S phase conditions. Despite concerns raised, there is no clear evidence to date that naked DNA vaccines can integrate. Gel-purified genomic DNA isolated from many tissues (including germline) following intramuscular injection of plasmid DNA and assayed by PCR has found no evidence of integration to a detection sensitivity of one copy per μg DNA (Ledwith *et al.* 2000). This rate is at least three orders of magnitude lower than the theoretical frequency of spontaneous gene inactivating mutations (Nicolas *et al.* 1995). Thus for plasmid DNA vaccines delivered by intramuscular injection, the risk of mutation due to integration

appears to be negligible. Other methods of plasmid DNA delivery (including gene gun or electroporation) or use novel formulations or adjuvants which increase the efficiency of cell transfection could potentially influence the integration frequency.

1.2.8.2 Potential for induction of immunological tolerance or autoimmunity

Most vaccines intended for human use are administered to infants and young children. Due to the immaturity of their immune system, there is a potential risk that exposure to DNA vaccines during the neonatal period may induce tolerance rather than immunity. Since protein encoded by a DNA vaccine is produced endogenously and expressed in the context of self-MHC, the potential exists for the neonatal immune system to recognise the encoded antigen as "self", resulting in tolerance rather than immunity. Consistent with this possibility, in one report a plasmid DNA vaccine encoding the circumsporozoite protein of malaria (CSP) induced long-lasting tolerance when administered to newborn but not adult mice (Ichino *et al.* 1999). Co-administration of plasmid DNA encoding GM-CSF prevented the development of neonatal tolerance in this model (Ishii *et al.* 1999). The simplest explanation appears to be that although CpG motifs associated with plasmid DNA are responsible for activation of DCs, additional maturation signals are required to optimise the response to certain DNA vaccines in neonates. Nevertheless, the majority of studies performed to date in rodents and non-human primates have demonstrated induction of significant cellular and humoral immunity in neonates (Manickan *et al.* 1997; Sarzotti *et al.* 1997). Increasing the content of CpG oligonucleotides or co-administration of plasmid DNA encoding Th1 driving cytokines (IL-12 or IFN-γ) has augmented TH1 responses where additional DC maturation signals

were found to be required in neonates (Kovarik *et al.* 1999). Overall, neonatal DNA vaccines rather than proving tolerogenic are substantially more effective in inducing Th1 responses than conventional vaccines.

Another theoretical concern is that autoimmune responses could occur as a result of immune-mediated destruction of plasmid DNA transfected cells expressing antigen. Cytolysis of antigen expressing cells would release self-antigens for uptake and presentation by DCs, theoretically capable of initiating autoreactive T and B cell responses. However, presentation of self-antigen occurs during normal cell turnover and in the course of viral and bacterial infections, without pathologic sequelae. In this respect. It appears unlikely that DNA vaccines would pose any greater risk in initiating organ-specific autoimmunity than conventional viral or bacterial vaccines.

1.2.8.3 Potential for the induction of anti-DNA antibodies

Concerns that DNA vaccines may promote the development of auto-antibodies to double stranded DNA (believed to be the hall mark of autoimmune disease such as systemic lupus erthematosus), arises from a series of observations. Bacterial DNA has been reported to induce anti-double stranded DNA antibodies in normal mice and to accelerate the development of autoimmunity in strains of genetically predisposed to lupus disease (Gilkeson *et al.* 1993). Bacterial CpG can also stimulate the production of IL-6 and block apoptosis of activated lymphocytes (Krieg *et al.* 1995; Klinman *et al.* 1996), mechanisms that may facilitate the persistent activation of B cells and predisposition to the development of SLE. Although an increase in IgG anti-DNA secreting cells has been

reported immediately following repeated immunisation of normal mice with plasmid DNA, the increases were small when compared to the spontaneous production of auto-antibodies reported in lupus-prone mice (Mor *et al.* 1997). Furthermore, the transient increase in serum IgG anti-DNA reported did not result in the development of disease in normal mice or accelerate disease in lupus prone strains of mice (Mor *et al.* 1997). While the possibility remains that a subset of DNA vaccines encoding determinants cross-reactive with self antigens may stimulate an autoimmune response, current data suggests that the level of anti-DNA antibody induced by plasmid DNA is unlikely to induce systemic autoimmune disease.

1.2.9 Clinical Trials

DNA vaccines have entered the clinic for initial safety and immunogenicity testing in humans. To date, the potency of the immune responses have been disappointing; nevertheless, humoral and cellular have been observed (Roy *et al.* 2000; Calarota *et al.* 2001). Clinical trials of DNA vaccines have been performed or are underway for various diseases including cancer, influenza, hepatitis B, HIV and malaria.

1.2.9.1 HIV

Phase I clinical trials were initiated to evaluate the safety and immunogenicity of HIV-1 env/rev DNA constructs in HIV sera-positive and low risk sera-negative individuals (Calarota *et al.* 1998; MacGregor *et al.* 1998; Boyer *et al.* 2000; MacGregor *et al.* 2000). Naïve healty volunteers who received the highest dose of DNA vaccine demonstrated antigen-specific lymphoproliferative responses and antigen specific production of IFN-γ and β-chemokines (Boyer *et al.* 2000), but these responses were weak and did not persist.

In HIV sera-positive individuals, a HIV-1 env/rev DNA vaccine construct boosted the env specific antibodies. However, no consistent effect was observed on cellular responses to HIV. Another phase I clinical trial evaluated HIV regulatory genes, such as rev, nef and tat. Immunisation of infected persons with these genes enhanced cellular responses but produced no consistent changes in lymphocyte subsets or viral load (Calarota *et al.* 1998; MacGregor *et al.* 1998; MacGregor *et al.* 2000; Calarota *et al.* 2001). The DNA vaccines were well tolerated in doses from 20µg up to 2500µg. No significant local or systemic reactions were observed, and no participant dropped out of the study (Le *et al.* 2000; MacGregor *et al.* 2000).

A phase I trial of an experimental HIV vaccine that includes an HIV A subtype gag gene and more than 20 epitopes of DNA encoding regions of HIV proteins is in progress in Kenya (Allen *et al.* 2000). This it he first component of a prime boost vaccination strategy and will be followed by a second vaccine using modified Vaccinia virus as a vector (Reynolds *et al.* 1981; Allen *et al.* 2000). Another phase I clinical trial was recently started in infected and uninfected persons to directly compare vaccines in which the gene is delivered as naked DNA or by attenuated adenovirus (Cohen 2001).

1.2.9.2 Hepatitis B

A DNA vaccine against hepatitis B virus was evaluated for safety and immunogenicity in a phase I clinical trial involving naïve healthy volunteers. A gene gun was used to propel the DNA into the skin. The hepatitis B DNA vaccine was found to be safe, well tolerated

and immunogenic (Roy *et al.* 2000). All of the volunteers developed protective antibody responses of at least 10mIU/ml. The vaccine induced specific $CD8^+$ T cell responses in the volunteers who were positive for HLA class I A2 allele. The antigen specific CD8+ T cells bound HLA-A2-hepatitis B surface antigens 335 to 343 tetramers, secreted IFNγ and lysed target cells presenting a hepatitis B surface antigen epitope (Roy *et al.* 2000).

1.2.9.3 Malaria

In a phase I clinical trial of malaria DNA vaccines, three intramuscular injections of a *Plasmodium falciparum* circumporozoite construct induced antigen-specific, $CD8^+$ T cell-dependent cytolytic T lymphocytes. The cellular responses were directed against multiple epitopes and were restricted by six HLA class I alleles. In the same study, despite induction of the excellent cytolytic T cell responses, DNA vaccination failed to induce detectable antigen-specific antibodies in the participants (Le *et al.* 2000).

In a study investigating the safety and immunogenicity of DNA and modified vaccinia virus Ankara (MVA) candidate vaccines, each encoding the malaria DNA sequence multiple epitope-thrombospondin related adhesion protein (ME-TRAP), against Plasmodium falciparum. DNA ME-TRAP and MVA ME-TRAP when immunised into naïve and malaria exposed individuals were safe and immunogenic for both $CD4^+$ and $CD8^+$ T cells induction (Moorthy *et al.* 2003).

1.3 Mucosal Immunity

The mucosal surfaces of the gastrointestinal and respiratory tracts represent the principal portals of entry for most human pathogens. Thus represents a critical component of the mammalian immunological repertoire. These include the gut-associated lymphoid tissue (GALT), the bronchoepithelium and lower respiratory tract (BALT), ocular tissue, upper airway, salivary glands, tonsils and nasopharynx (NALT), larynx (LALT), middle ear cavity, male and female genital tracts, mammary glands and the products of lactation.

The mucosal surfaces are protected from pathogens by non-specific mechanisms such as the production of mucus, which forms a physical barrier, and substances such as lysozyme, lactoferrin and lactoperoxidase, which kill micro-organisms or inhibit their replication. In addition, the MALT protects the mucosa from exogenous aggression by discriminating between fundamental uptake of food or harmless antigens, commensal micro-organisms and dangerous pathogens. Thus is able to mount both immunological and mucosal tolerogenic responses.

The mucosal immune system consists of specialised local inductive sites, the organised mucosa associated lymphoid tissue (O-MALT), and widespread effector sites, diffuse mucosa-associated lymphoid tissue (D-MALT), both of which are separated from mucosal surface antigens by epithelial barriers (Kraehenbuhl and Neutra 1992). The first step in the induction of a mucosal immune response is the transport of antigens across the epithelial barrier. Following antigen processing and presentation in inductive sites, IgA committed antigen-specific B lymphoblasts proliferate locally and then migrate via the

bloodstream to local and distant mucosal and secretory tissues. There they differentiate primarily into polymeric IgA producing plasma cells, an important component of D-MALT (Kraehenbuhl and Neutra 1992). Dimeric or polymeric IgA antibodies are transported across epithelial cells into glandular and mucosal secretions via receptor mediated transcytosis (Apodaca *et al.* 1991).

1.3.1 IgA

Dimeric IgA is the principal class of immunoglobulin found in mucosal and exocrine gland secretion. IgA secreting B cells can be activated in both T cell dependent and T cell independent mechanisms. The latter is important in producing antibodies that are protective against the commensal bacteria that populate the gut and probably represent a primitive form of specific immune defence (Macpherson *et al.* 2000; Fagarasan *et al.* 2001). dIgA is composed of two monomeric IgA subunits and a polypeptide J chain, although trimers and tetramers are also found.

Following secretion, dIgA bind to the polymeric immunoglobulin receptor (pIgR) that is located on the basolateral surface of the epithelial cells that form the mucosa. The pIgR is a transmembrane protein with an extracellular ligand binding region. After synthesis in the endoplasmic reticulum and exit from the Golgi pIgR is a delivered directly from the trans-Golgi network to the basolateral surface where it binds dIgA. The pIgR-dIgA complex is then endocytosed and transported through a series of endosomal compartments across the cell to the apical surface. En route or at the apical surface, pIgR is proteolytically cleaved and the extracellular binding domain that is bound to dIgA is released into the mucosal secretions. This cleaved extra-cellular domain of the receptor is

known as the secretory component (SC). Secreted dIgA in association with the SC is known as secretory IgA (sIgA). Knockout mice that lack pIgR have a significant defect in dIgA secretion, although a small but significant amount of dIgA is still formed in bile, faeces and intestinal content, which indicates that other pathways for dIgA secretion exists (Shimada *et al.* 1999).

1.3.2 Epithelial surfaces

Epithelial linings of different mucosal surfaces differ dramatically. Multilayered squamous epithelia line the oral cavity, pharynx, esophagus, urethra and vagina. The intestinal mucosa is covered by only a single cell layer and the airway lining varies from pseudostratified to simple epithelium. These diverse epithelia are not impenetrable barriers, but are cell assemblies that control cross-talk between the exterior and interior using multiple antigen sampling strategies. In stratified and pseudostratified epithelia, antigen processing dendritic cells serve as motile "scouts" that move into the epithelium, obtain samples of luminal antigens and migrate back to local or distant organised lymphoid tissues. In simple intestinal and airway epithelia where the intracellular spaces are sealed by tight junctions, specialised epithelial M cells deliver samples of foreign material by transepithelial transport from the lumen to organised lymphoid tissues within the mucosa.

1.3.3 M Cells

M-cells represent specialised epithelial cells that actively transport soluble and particulate matter across the epithelium (Gebert *et al.* 1996). Thereby, performing a "sampling" of luminal antigens so that cells of the immune system come into contact with potential

pathogens (Neutra *et al.* 1996). M cells thus combine two important functions maintenance of the barrier and initiation of mucosal immune reactions. However, organisms such as Poliovirus, Rotavirus and Salmonellae can subjugate this machinery (Neutra *et al.* 1996).

The transepithelial transport of substances by M cells is preformed by fluid phase or receptor-mediated endocytosis at the apical membrane, transport in vesicles across the cytoplasm and exocytosis to the basolateral membrane (Neutra *et al.* 1987). The basolateral surface is deeply invaginated to form large intraepithelial "pockets" into which transcytosed particles and macromolecules are delivered (Neutra and Kraehenbuhl 1992). This unique structural modification is supported by an extensive array of intermediate filaments and is lined by a distinctive "pocket domain" of the plasma membrane (Neutra *et al.* 1987). The pocket and its content of immigrant cells is a cardinal feature of the M cell. M cells also have basal processes that extend 10μm or more into the underlying lymphoid tissue where they could make direct contact with lymphoid or antigen presenting cells (Giannasca *et al.* 1994).

The immigrant cells in M cell pockets have been identified by immunocytochemistry of intestinal Peyer patches of rodents, rats and humans (Ermak and Owen 1986; Ermak *et al.* 1990). In all species, both B and T lymphocytes were present along with a small number of macrophages. Most of the T cells were CD4+, and none displayed the gamma/delta T cell receptor of villus intraepithelial lymphocytes (IELs). Human M-cell associated T cells displayed the marker antigen CD45RO typical of memory cells, although in some

specimens naive T cells were observed (Farstad *et al.* 1994). B cells in the pocket expressed the "naive" cell marker CD45RA along with HLA-DR suggesting that the M cell pocket is a site of interaction between T cells and antigen presenting B cells. The B cells contained IgM but neither IgG nor IgA, suggesting that B memory cells and initial B cell differentiation may also occur here. Since the B cells in the M cell pockets are the same types as the subepithelial B cells associated with the underlying follicle, it has been suggested that B lymphoblasts traffic into the M cell pocket allowing for continued antigen exposure, thus extending and diversification the immune response (Farstad *et al.* 1994).

1.3.3 GALT

One of the best studied sites of mucosal immunology is the gut associated lymphoid tissue (GALT). Lymphoid cells are found in three regions within this tissue. The outer mucosal epithelial layer contains IELs. The majority of these lymphocytes are CD8+ T cells that express γδ T-cell receptors. The lamina propria, which lies under the epithelial layer, contains large numbers of B cells, plasma cells, activated T_H cells, and macrophages in loose clusters. Finally, within the sub-mucosal layer of the intestinal lining are nodules consisting of 30-40 organised lymphoid follicles, called Peyer's patches.

The common feature of GALT includes an epithelial surface containing M-cells overlying organised lymphoid follicles. Mucosal epithelium has a unique structure, and in addition to M cells, it also contains mucin producing glandular cells. lymphocytes, plasma cells, dendritic cells and macrophages. The dendritic cells are present in different

components of the common mucosal immune system, including both the organised lymphoid tissue and the mucosal epithelium. These cells can be strongly associated with potentiation of the immune response whether by inducing active immunity (Liu and MacPherson 1991; Liu and MacPherson 1993) or tolerance (van Ginkel *et al.* 2000).

Following exposure of an antigen and its uptake via M-cells, there is a variable degree of activation of T cells, DCs and B cells, especially of the IgA isotype. Activation of T cells results in the release of a number of distinct cytokines and chemokines from different T-cell subsets and recognition of antigenic epitopes including MHC class I and II molecules.

1.3.2 NALT

NALT is similar in its morphological and functional characteristics to other mucosal tissues such as the GALT and BALT; however, it has better developed lymphoid follicles, with marked intraepithelial infiltration by lymphocytes and dendritic cells. NALT resembles Peyer's Patches with regards to ontogeny although PP appear shortly before birth, earlier than NALT. This may possibly be due to a more central role for the PP or may simply result from earlier exposure of the gut to foreign antigens. The activated appearance of both NALT and PP is suggestive that they are the main components in the defense of the respiratory tract and gut respectively. The functions of NALT and PP may differ, with the PP being the central tissue in the induction of secretory Ig synthesis, while the lymphoid tissue in the respiratory tract appear to be more involved in cellular responses. The respiratory tract can be broken down into NALT

and BALT, and appear to differ in their state of activation and in the number of B cells that express membrane IgA. BALT more closely resembles PP in the frequency of these cells, where as in NALT, they are quite rare (Butcher *et al.* 1982; Plesch 1982). The follicular areas are organised into B cells and intrafollicular (T-cell) areas of approximately similar size. In the mouse, lymphoid aggregates called the O-NALT is located on the palate at the entrance to the nasopharyngeal duct, and the less well organised diffuse lymphoid tissue lining the nasal passages (D-NALT) (Liang *et al.* 2001). Particulate antigens may be removed quickly from the nasal mucosa by the mucociliary system. However, when the antigens succeed in adhering to the epithelium they are taken up by M cells. The NALT M cells appear to be identical to those in Peyer's patches and BALT and are involved in similar immunological functions including antigen uptake and subsequent mucosal immune responses to specific antigens (Park *et al.* 2003).

The nasal mucosa is drained by the superficial cervical lymph nodes (CLN) which in turn drain to the posterior CLN. However, large doses of antigenic material may drain directly to the posterior lymph nodes. Although the nasal mucosa drains to the superficial cervical lymph nodes, NALT drains preferentially to the posterior cervical lymph nodes (Tilney 1971; Koornstra *et al.* 1991). Antigenic stimulation of the mucosal immune system is known to induce specific local immune responses, notably the production of secretory immunoglobulins and to result in specific systemic tolerance. A study examining the repeat intranasal inoculation of ovalbumin observed that tolerance was established (Holt *et al.* 1987). The superficial CLNs that drain the nasal mucosa are the site of suppressor

T-cell induction and activation, and may play a role in the generation of tolerance. Single intranasal immunisation evoked almost no response in either serum or in BALT. NALT. posterior and superficial CLNs and spleen. A second intranasal immunisation with a soluble T-cell dependant antigen resulted in a distinct response in the serum and in the draining posterior CLN only (Holt *et al.* 1987).

1.4 Hypothesis to be tested

The need to improve the efficacy of plasmid DNA immunisation is still of prime importance. As the immune system is constantly being elucidated, potential candidates for genetic adjuvants or delivery vehicles are being identified. To overcome the hurdle of the dose per Kg of DNA required to initiate immunity to a specific antigen, new techniques are investigated to enhance the immune responses. In this study we have examined the use of different encapsulation methods as well as the use of a genetic adjuvant to increase the efficacy of plasmid DNA delivery.

Chapter 2 – Materials and Methods

2.1 Plasmid DNA Preparation

2.1.1 Propagation of Plasmid DNA

E.coli transfected with the plasmid of interest (Table 1) were streaked from a glycerol stock onto an LB Agar plate (recipe Appendix 1), containing the appropriate antibiotic selection (Table 1). Streaked plates were grown O/N and all subsequent cultures inoculated from single colonies not more than 48 hours old.

2.1.2 Mini preparation of plasmid DNA

A single colony (Section 2.1.2) was inoculated into 1ml of Terrific Broth, and grown for 4 hours at 37°C. The bacteria were pelleted at 1650g in GSA rotor (RC5C, Sorvall Instruments). The pellet was resuspended in 100µl of Solution 1 (see Appendix 1, containing 5µl of 10mg/ml RNAse A), incubated for 5 minutes at room temperature, followed by addition of 200µl of Solution 2, and mixing the contents by gently inverting the tubes (5 or 10 times). After a 5 minute incubation on ice, 150µl of solution 3 was added and mixed gently (by inverting the tube 5-10 times) and incubated for 15 mins on ice. Tubes were centrifuged at 16K g for 5 mins in a Biofuge pico (Heraeus), supernatants were removed and extracted with 400µl phenol-chloroform. DNA was precipitated by the addition of 2 volumes of ethanol and 0.1 volume NaAc for 1 hour at -20°C, and pelleted at 16K g. Pellets were ethanol washed, air dried and resuspended in TE. The optical density was determined at 260nm and 280nm.

2.1.3 Maxi Preparation of Plasmid DNA.

An overnight culture seeded from a single colony (section 2.1.2) was diluted 1 in 1000 into 1L of 2xYT and grown until the absorbance was 1.2 at 600 nm. Cultures were centrifuged at 1650g, for 15 minutes at 4°C in a GSA rotor (RC5C, Sorvall Instruments), and the pellets resuspended in 18mls Solution 1 (see Appendix 1) for 10 minutes (all procedures were performed at room temperature unless otherwise stated) followed by the addition of 40mls Solution 2 (see Appendix 1). After 10 mins, 20ml of ice cold Solution 3 (see Appendix 1) was added and incubated on ice for 10 mins and centrifuge at 8000g for 30 minutes at 4°C in SS-34 rotor (RC5C, Sorvall Instruments). DNA was precipitated with 0.6 volume of propanol (BDH) and centrifuge for 20 minutes, at 9500g in the SS-34 rotor. Supernatants were decanted and the pellet washed with 70% Ethanol and air-dried for 5 minutes. The pellets were resuspended in TE (see Appendix 1), containing 1mg/ml of caesium chloride (ICN). To this 0.6 volume of ethidium bromide was added, and then the solution was centrifuged at 8000g for 20 minutes at 4°C in SS-34 rotor. The supernatants were transferred to sealable Ultra Crimp Beckman tubes, balanced, sealed and centrifuged for 16 hours at 65,000rpm in T8-90 rotor with a Discovery 100 Ultracentrifuge (Sorvall). The caesium chloride gradient caused bands to be formed, with the upper band being nicked plasmid DNA, and the lower being supercoiled plasmid DNA The lower band was harvested using a needle and syringe, transferred to a new centrifuge tube, balanced, resealed and spun for another 16 hours at 65,000rpm. The ethidium bromide was separated from the plasmid DNA by repeated extractions with an equal volume of Butan-1-ol (BDH) vortexing and centrifuging at 1500rpm for 3 minutes until it was visibly removed from the upper aqueous layer. Any remaining organic

solvents and the CsCl were removed by dialysis over 48 hours with at least 4 changes in dialysis buffer (see Appendix 1). Protein and organic contaminants were removed by phenol chloroform extraction, centrifuged for 6 minutes at 3500 rpm (2537g) in a RTH750 rotor (RT7 Plus Centrifuge, Sorvall). DNA was precipitated with 2.5 volume 100% Ethanol (BDH) at -70°C (for a minimum of 2hr) followed by centrifugation at 3500rpm for 10 mins at 4°C. The DNA was washed with 70% ethanol, and vacuum dried using a Concentrator 5301, (Eppendorf). The pellet was resuspended in pyrogen free water and the concentration of and purity of the plasmid DNA was determined by spectrophotometric analysis at 260 and 280nm respectively using a Ultrospec 3000 (Pharmacia Biotech). An absorbance of 1 at 260nm is equal to 50ug/ml of double stranded DNA, and DNA preparations were rejected if the OD 260/280 ratio was below 1.8.

2.1.4 Restriction Enzyme Digestion of plasmid DNA

Restriction enzyme digestion was used to verify the correct cDNA insert in each preparation of plasmid DNA. Restriction enzyme digestions were performed using the NEB system. 1µg of DNA, was added to 2µl 10x buffer (NEB), 10 units of enzyme (1 unit [NEB]), the volume was made up to a total of 20µl with distilled water, and digested for 1 hour at 37°C. The plasmid was visualised on a 1% agarose gel (1% agarose in TAE, with ethidium bromide at 2ug/ml) using a Flowgen Submarine electrophoresis apparatus. DNA was electrophoresed for 40 minutes at 80volts, and visualised using a UV Transilluminator (UVP).

2.2 Transfections of Eukaryotic cells

2.2.1 Plasmids under investigation

Plasmid backbone	Insert	Antibiotic Resistance	Company
g-Wiz	SEAP	Kanamycin	Gene Therapy Systems
	GFP	Kanamycin	
	HIV-Gag	Kanamycin	
VR	SEAP	Kanamycin	Vical
	HIV-Gag	Kanamycin	
	Luciferase	Kanamycin	
pcDNA3.1+	CCL20	Ampicillin	Invitrogen
	HIV-Gag	Ampicillin	
pTRE-Shuttle	HIV-Gag	Ampicillin	Clontech
PCi	SEAP	Kanamycin	Promega

Table 1 – List of the plasmid DNA used in this study, including the backbone vector, the insert, the antibiotic resistance and the company where the vector was purchased.

2.2.2 Cell transfections with plasmid DNA

HEK293T (ECACC no: 85120602), a human embryonic kidney cell line was transfected to determine the transduction efficiency of naked or complexed plasmid DNA preparations. Cells were plated at a density of 1 x 10^5 cells per well in a 24 well plate (Costar) in DMEM (see Appendix 1) subsequently called DMEM+, and incubated at 37°C in 5% CO_2. After 16 hours the supernatant was removed, and the cell monolayer was washed with phosphate buffered (PBS – see Appendix 1) The PBS was removed and 300µl of either naked DNA, DNA/lipid complexes or DNA/peptide complexes was

added very slowly to each well. The cells were incubated at 37°C for 2 hours, and then an equal volume of 300µl 2x concentration of DMEM+ was added per well. and cultured for a further 48hours.

2.2.3 Lipids under investigation in the study

The lipids used in the study were kindly supplied by Vical Inc, San Diego.

Lipid Name	Chemical composition
DMRIE:DOPE	Dimyristyloxypropyl-dimethyl-hydroxyethyl ammonium bromide/dioleoyl-phosphatidyl-ethanolamine
GAP-DLRIE	Aminopropyl-dimethyl-*bis*-dodecyloxy-propanaminium bromide
GAP-DMORIE:DpyPE (Vaxfectin)	Aminopropyl-dimethyl-myristoleyloxy-propanaminium bromide/diphytanoylphosphatidyl-ethanolamine

Table 2 – List of cationic lipids used in this study, and there chemical structure.

2.2.4 Complexing of DNA with lipid

The amount of transfection agent to DNA was calculated in molar ratios. The molarity of DNA is calculated based on the average weight of a single nucleotide; 1 mole is equivalent to 333g of DNA in 1L. Therefore, DNA at 1mg/ml will have a concentration of 3.33mM. The lipids, GAP-DMORIE:DpyPE, DMRIE:DOPE, and Gap:DLRIE were formulated at 1.5mM in double distilled water. A well in a 24 well plate, is typically transfected with 1µg of DNA. Therefore, at a ratio of 1:1, 1ug of DNA is complexed with 2µl of lipid (calculation = (DNA concentration/lipid concentration) when DNA = 1mg/ml for 1ug of DNA). To complex, lipids were diluted in saline, and then added dropwise to an equal volume of DNA which was also diluted in saline. The two were mixed by flicking the tube after each drop, not by vortexing. After the two components were

thoroughly mixed together they were incubated for 30minutes at room temperature. The volume was then increased to 300µl (the amount added to a 24 well plate) with DMEM without any additives.

2.2.5 Peptides investigated in this Study

The peptides were synthesised by Cambridge Research Biochemicals to a 99% purity as determined by HPLC.

Peptide name	Sequence	Molecular Weight (Daltons)
Antennapedia-K16	RQIKIWFQNRRMKWKKGGGKKKK KKKKKKKKKKKKK	4468.7
Antennapedia	RQIKIWFQNRRMKWKK	2417.9
Polylysine-Molossin	ICRRARGDNPDDRCTGGGKKKKKK KKKKKKKKKK	3797

Table 3 – List of peptides investigated in this study, their amino acid constituents and their molecular weight.

2.2.6 Complexing DNA with peptide

Peptides were dissolved within PBS, 10mM Tris Saline, 1mM HEPES Saline, Saline or DMEM (without additives), at a concentration of 1mg/ml, which is equivalent to 0.22mM Antennapedia-K16, 0.26mM Polylysine-molossin and 0.41mM Antennapedia. In a 24 well plate, each cell was transfected with 1µg of plasmid, in a total volume of 300µl.Complexes were formed in 15ml conical tube (Corning), where plasmid was diluted in DMEM without additives. The peptide was added at the required concentration during vortexing of the plasmid solution. For example, at a 1:1 molar ratio of

DNA:antennapedia-K16, the DNA is 3.33mM, the peptide is 0.22mM. So for 1μg of DNA, you would need 13.6μg of peptide, and for a 6:1 ratio, you would need 2.7μg of peptide. The DNA:peptide complex was incubated for 30minutes at room temperature, and was then added to the cells and reincubated at 37°C. .After 2 hours, the supernatant was removed, the cells were washed, and 1ml of DMEM+ was added per well.

2.3 Reporter Assays

2.3.1 Generation of Standard Curves.

Standard curves were generated with the appropriate recombinant enzyme. A range of standards were created, for luciferase, the range was log dilutions starting at 10ng per ml to 1pg per ml of recombinant luciferase (Sigma), for SEAP, the range was halving dilutions from 1ug per ml to 7.8125ng.of recombinant SEAP (Calbiochem). Each dilution was done in duplicate, the averages were plotted, and a standard curve was generated. The equation for the curve was calculated, and experimental values were obtained.

2.3.2 Luciferase (reporter gene) assay of cell lysates.

Test samples were compared against a standard curve generated in each assay of known concentrations of recombinant luciferase (Sigma UK). Cell lysates were generated by adding 200μl of Cell Lysis buffer (Promega, UK) to the cell monolayer in a 24 well plate. After 5 minutes, the cell monolayer was removed, and centrifuged at 13,000rpm ($15.8x10^3$g) for 2 minutes. 20μl of the cell lysate supernatant was added to 100ul of Luciferase Assay Reagent (Promega, UK). Luminescence was immediately measured for

1minute on a Turner TD20e luminometer. The concentration of luciferase in the samples was expressed as pg of luciferase protein, as determined from the standard curve.

2.3.3 Secreted Alkaline Phosphotase (SEAP) assay of supernatants.

Supernatants were heat inactivated at 56°C for 30mins, to remove endogenous SEAP. A standard curve was established using recombinant SEAP (Calbiochem) (Section 2.2.6). 50µl of supernatant or recombinant SEAP was added to 200µl of SEAP substrate (see Appendix 1). Readings were taken at 2, 5, 10, 15, 30 and 60 minutes at 405nm with a λ correction of 605nm with an Anthos 2010.

2.3.4 Flow Cytometric Analysis of Green Fluorescent Protein

At 48 hours following transfection with either gWiz-GFP or VR-GFP. Cells were washed with PBS, and followed by two washes with FACS (see Appendix 1), then fixed with 300µl 1% paraformaldehyde (PFA) (Sigma, UK). Cells were analysed with a Coulter flow cytometer, and data was processed using WinMDI v2.8 (Copyright Joseph Trotter).

2.3.5 HIV Gag flow cytometry

Cells were transfected with gWiz-Gag, Vical-Gag, pcDNA-Gag, and a pTRE-Shuttle. Cell monolayers were washed with PBS and removed from the wells using FACS buffer, spun down in LP4 tubes (LIP) 500g, at 4C for 5 mins in a H6000A rotor (RC3C, Sorvall Instruments). Cell were fixed by resuspension in 1% PFA in PBS for 15 minutes, washed twice with FACS buffer, and then permeablised with 0.2% Saponin in PBS for 15 minutes at room temperature. After 3 washes with FACS buffer, a murine anti-human-Gag antibody (1/100) in 100ul was incubated with the cell suspension for 1 hour on ice. After 4 washes with FACS buffer, a goat anti-mouse-PE (1/50) was incubated with the

cells for 1 hour on ice. Cells were washed 5 times with FACS buffer. and resuspended in 300ul FACS buffer. Cells were analysed on an EPICS XL MCL (Coulter), and data was processed using WinMDI v2.8 (Copyright Joseph Trotter).

2.3.6 SDS PAGE and Western blot of transfected cell lysates and supernatants

Cell lysates (containing both cytoplasmic and membrane fractions) were prepared with an equal volume of reducing sample buffer (See Appendix 1) and boiled for 3minutes at 100°C. 20ul of boiled samples were loaded per well of a 10% polyacrylamide gel (See Appendix 1) with a 5% stacking gel (See Appendix 1). Samples were electrophoresed using a Biometra P25 power pack, at 80 volts constant voltage for 2 hours. The separated proteins were electroblotted onto Hybond C-Super nitrocellulose membrane (Amersham UK) at 240mA for 2 hours using a Transphor Electrophoresis Unit (Hoefer, UK) in transfer buffer (see Appendix 1). The membrane was washed twice in Tris Buffered Saline pH 7 (TBS – see Appendix 1) containing 0.1% Tween-20 (called TBS-T) for 5 minutes and blocked overnight at 4°C in TBS-T containing 5% FCS. A primary antibody, anti-murine CCL20 (R&D Systems) was diluted 1/500 and preincubated in TBS-T with 5% FCS for 1 hour, and then incubated with the membrane for 2 hours. Unbound antibody was removed by washing 3 times for 10 mins in TBS-T. Bound antibody was detected by incubating the membrane with a Horse Radish Peroxidase (HRP) conjugated antibody (Sigma, UK), diluted 1/1000 in TBS-T containing 5% FCS for 2 hours, followed by 4 washes in TBS-T, the membrane was developed with Enhanced Chemi-Luminescence (ECL) reagents (Amersham, UK) and exposed onto Hyperfilm-ECL (Amersham, UK) for 10 seconds, 30 seconds, 1 minute, and 2 minutes.

2.3.7 Transwell Chemotaxis Assay

The chemotactic ability of bone marrow derived DCs (BMDCs) was analysed using ChemoTx system according to the manufacturer's instructions (96-well ChemoTx chamber; Neuro Probe, Inc.). In brief, BMDCs were resuspended in RPMI 1640 supplemented with 1% FCS and 25 mM Hepes. Supernatants from HEK293 cells transfected with pcDNA3.1-CCL20 for 48 hours or recombinant CCL20 were placed in the lower chamber, and a filter with 5-μm pore size was placed on top. Aliquots of $2x10^4$ cells/well were applied to the filter's top surface, and the plate was incubated at 37°C in 5% CO_2 for 4 h. The cells migrating to the bottom chamber were counted with an inverted microscope in five or more non-overlapping fields (magnification 40).

2.3.8 Calcium Flux Assay

Calcium Flux assays were carried out upon Rat splenocytes and HEK293T cells stably transfected with either human CCR6 or murine CCR6. Rat splenocytes were harvested from a Lewis Rat, and homogenised into a single cell suspension by grinding through a sieve in RPMI+ (see Appendix 1). To remove adherent cells, the splenocytes were passed through a nylon column, leaving the T and B cell populations. HEK293 cells stably expressing hCCR6 or mCCR6, or the rat splenocytes were suspended in 1×10^7/ml in DMEM with 10% foetal calf serum. The cells were loaded with 3 μM Indo-1 acetoxymethyl ester (Molecular Probes) at room temperature in the dark for 45 min. The loaded cells were washed and resuspended in Hank's balanced salt buffer with 1% foetal calf serum at the same concentration. Calcium mobilization of 1×10^6 cells was measured in 2 ml of Hanks' balanced salt solution with 1.6 mM $CaCl_2$ in a continuously stirring acrylic cuvette at 37°C in a Photon Technologies spectrofluorimeter (Princeton). The

fluorescence was monitored as ratio of emission at 405 and 483 nm at an excitation wavelength of 350 nm. The chemokines examined were added at the concentrations of 200nM or 500nM recombinant human or murine CCL20 or 20nM SDF-1α.

2.4 *In vivo* studies

2.4.1 Mice

All procedures were performed with seven to eight week old female Balb/c mice (Harlan Olac, Bicester, UK), with a weight not less than 18g. Animal care throughout the study was in accordance with UK Home Office guidelines.

2.4.2 Intranasal immunisation.

Cytofectins, GAP-DMORIE:DpyPE or DMRIE:DOPE were complexed with plasmid DNA to increase the efficiency of DNA uptake for intranasal delivery. Typically, doses of plasmid DNA ranged from 40μg to 100μg with complexing ratios ranging from 4:1 to 16:1 molar ratios. In accordance to Home Office guidelines, the maximum volume administered was 60μl, over a minimum of 2 hours. Complexing was achieved by a similar method as in section 2.2.4. Briefly, plasmid DNA and lipid were each diluted in saline to 50% of the final volume required for the immunisation. The lipid was added to the DNA dropwise, with thorough mixing by flicking the tube after each addition, thus leaving a cloudy solution with a minimal amount of precipitation. Depending on the overall volume required for immunisation, a minimum of 5μl to a maximum of 12μl was applied to the nares at one time, with a maximum of 5 applications allowed. Dosage to the nares was performed as slow as possible, to allow coating and absorbance of the nasal tissue.

2.4.3 Intramuscular Immunisation.

Plasmid DNA was made up to a maximal concentration of 1mg/ml in saline. The mice were anaesthetised with Halothane (Sigma), and kept anaesthetised by the use of a mask. 50µl of the plasmid DNA solution was injected into the calf muscles of the two hind limbs.

2.4.4 Subcutaneous Immunisation

50µl of DNA at 1mg/ml in saline was inoculated by the subcutaneous route at the base of the tail.

2.5 Tissue Isolation

2.5.1 Generation of Bone Marrow Derived Cells

Femurs and tibiae from Balb/c mice were harvested, and the surrounding muscle was dissected from the bone. Both ends of the bone were cut, and the marrow was flushed through the bone with RPMI+ (see Appendix 1) using a needle. Clusters within the bone marrow were disintegrated by vigorous pipetting. Bone marrow was seeded at $2x10^6$ cells per 100mm dish in 10mls of RPMI+ containing 50µM 2-mercaptoethanol (Sigma) and 20ng/ml recombinant GM-CSF (Peprotech) and cultured for 6 days at 37°C.

2.5.2 Nasal Associated Lymphoid Tissues.

The head was severed from the bodies and the skin and the eyes were removed. The cranium from just behind the eyes, and the brain, were removed, as was the lower jaw,

leaving the nasal passages. Extra bone was trimmed away, leaving a minimal amount of bone and tissue.

2.5.3 Luciferase expression in NALTS.

The isolated bony nasal cavities were ground to a powder using a pestle and mortar under liquid nitrogen and dissolved in cell lysis buffer, to give a 1mg/ml concentration (determined by weight of tissue). After vigorous vortexing, the lysates were incubated on ice for 5 minutes, centrifuged at 16K g for 5 minutes and supernatants were collected. 20µl of supernatant was added to 100µl of Luciferase Assay substrate (Sigma). Luminescence was immediately measured for 1 minute on a Turner TD20e luminometer. The concentration of luciferase in the samples was expressed as pg of luciferase protein, as determined from the standard curve (2.2.6).

2.5.4 Flow cytometric analysis of NALT tissue.

Cell Surface Molecule	Clone No	Isotype	Conjugate	Conc/106 cells	Company
CD11c	HL3	IgG	PE	0.5µg	Pharmingen
CD11b	M1/70	IgG_{2b}	Biotin	0.5µg	Pharmingen
CD16/32	2.4G2	IgG_{2b}	Pure	0.5µg	Pharmingen
MHC class II	NIMR-4	IgG	Biotin	0.5µg	Southern Biotechnology Associates Inc.
Streptavidin	-	-	Cy-Chrome	0.5µg	Pharmingen
CCR6	-	IgG	FITC	1/20 dilution	Kindly supplied by Dr C.Power, Serono Pharmaceuticals, Switzerland

Table 4 – Antibodies used in this study, with appropriate concentrations as used in all experiments.

The NALT was harvested by piercing the nasal cavities and scraping out all nasal tissue into RPMI+ (see Appendix 1). The tissue was disassociated with fine needles, into a cell suspension and cells were counted, maximally $6x10^5$ cells were recovered per mouse. $2x10^5$ cells were aliquoted into LP4 tubes (LIP) and washed twice with FACS buffer. Cells were blocked with antiCD16/CD32 (BD Pharmingen) at 1/100 dilution for 15 minutes, and primary antibodies were added in 100µl for 1 hour. Cells were washed three times with FACs buffer, and if required, a streptavidin secondary (1/100) was added 100µl per tube, and incubated on ice for 30minutes. Tubes were washed for a minimum of three times. Cells were analysed on an EPICS XL MCL (Coulter) flow cytometer, and data was processed using WinMDI v2.8 (Copyright Joseph Trotter).

2.5.5 Isolation and immunofluorescence staining of NALT DC.

The heads from experimental and control mice were collected following exsanguinations under terminal anaesthesia. Tissue was trimmed to the nasal cavity and cell suspension isolated by teasing through a sieve in RPMI+. Mononuclear cells were purified by centrifugation through a 75% and a 40% percol gradient. Cells from the interface were washed in FACs buffer counted and stained. The cells were incubated with anti CD16/CD32 receptor block (BD Pharmingen) at 1:100 for 15 minutes and then stained with CD11c biotin (BD Pharmingen) at 1:100 for 1 hour at 4°C. After 3 washes with FACs buffer, biotin labelled cells were revealed using streptavidin conjugated PE-Cy5

(BD Pharmingen) at 1:100 dilution. Cells were washed 5 times in FACs buffer to remove excess streptavidin, and then sorted based on their CD11c expression on a Mo-Flo high-speed multi-laser sorter (Cytomation). CD11c expressing cells were cytosmeared onto in situ PCR Glass slides (Perkin Elmer), and fixed in pre-chilled acetone at –70°C for 15minutes. Slides were washed in PBS, and then blocked with normal rabbit serum (Vector Laboratories) at 1:100 for 15 minutes, followed by staining with a rabbit anti-GFP FITC poly-clonal antibody (Sigma) (1:100) for 1 hour. Cells washed with PBS, and then examined by immunofluorescence camera (Photonic Sciences, Nikon)

2.5.6 RNA isolation from NALT

NALT was ground up to a fine powder under liquid nitrogen using a pestle and mortar and resuspended in 1ml TRIZOL (Invitrogen) for 50-100mg of tissue, and incubated for 15 minutes at room temperature, then centrifuged at 13,000rpm ($15.8x10^3$g) for 10 minutes. The supernatant was removed, and 0.2ml of chloroform (BDH) was added to 1ml of TRIZOL containing supernatant. Tubes were shaken vigorously for 15 seconds, incubated for 2 minutes at room temperature, and then centrifuged for 15 minutes at 13,000rpm ($15.8x10^3$g). The RNA from the aqueous phase, was removed to a fresh tube and precipitated with 0.5ml isopropanol (BDH) to 1ml of starting TRIZOL. Samples were incubated at room temperature for 10minutes, and then centrifuged at 13,000rpm ($15.8x10^3$g) for 10 minutes and then the RNA pellet was washed twice with 70% ethanol, and resuspended in Diethyl cyanophosphonate (DEPC) treated water (Sigma). RT-PCR was carried out as the ProSTAR First-Strand RT-PCR Kit (Stratagene). Briefly, 5µg of RNA was diluted into 38µl with DEPC treated water. To this 3µl of random primers (100ng/µl) was added, and mixed gently. The reactions were incubated at 65°C for 5

minutes, and then were allowed to cool until room temperature. to allow the primers to anneal to the RNA. Into this reaction, the following was added in order. 5µl of 10x first-strand buffer, 1µl of RNase Block Ribonuclease Inhibitor (40U/µl). 2µl of 100mM dNTPs and 1µl StrataScript reverse trascriptase (50 U/µl). The reactions were incubated at 90°C for 5 minutes, and then placed on ice for the PCR reaction. 3µl of the cDNA produced was placed in a separate tube, and the following was added in order. 10µl of 10x Taq DNA polymerase buffer, 0.8µl of 100mM dNTPs, 2µl of 10µM forward primers, 2µl of 10µM reverse primers, and the volume was made up to 99.5µl

Primer	Sequence
CCR6-Forward	GGTCTGTCTCAGAGCCCATC
CCR6-Reverse	ATTTTCGACGGTCTCACTGG
CCL20-Forward	CTTGCTTTGGCATGGGTACT
CCL20-Reverse	AGGAGGTTCACAGCCCTTTT
T7 promoter	TAATACGACTCACTATAGGG
HPRT-Forward	CGATGATGAACCAGGTTATGACC
HPRT-Reverse	CCTTTCCAGTTAAAGTTGAGAGATC

Table 5 – List of primers used in this study.

The reactions were heated to 91°C for 5 minutes, then cooled to 54°C for 5 minutes. before being pulsed centrifuged, and having 0.5µl of Taq DNA polymerase (5U/µl) added. Mineral oil was carefully overlaid on top of the reaction, and then placed onto the PCR block, for 30 cycles of 91°C for 1 minute, 54°C for 1 minute and 72°C for 2 minutes. On completion, the reaction was incubated at 72°C for 10 minute. The PCR

product was analysed on a 1% ethidium bromide stained agarose gel. and product sizes were identified using DNA markers (Stratagene).

2.6 Immunohistochemistry

2.6.1 Immunohistochemistry of tail sections.

50μg of plasmid DNA encoding CCL20 was injected subcutaneous into the base of the tail. After, 24, 36, 48, or 72 hours tails were harvested. and mounted on OCT. At least 3. 10 micron sections were analysed per time point, per tail. The area around the section was scored with a diamond pen. Sections were placed in acetone for 30 minutes and then washed in TBS. Within a humid chamber, the section was washed 3 times with TBS. Excessive liquid was removed from the sections and the antibodies diluted in TBS were applied to the scored area. The antibodies used were biotin conjugated- MHC class II (Southern Biotech, Clone: NIMR-4, Rat IgG) and CD11c (Pharmingen, Clone HL3. Armenian Hamster) diluted 1:100. After a 1 hour incubation. the section was washed with TBS, and the section was incubated with a 1:1000 Streptavidin-Alkaline phosphatase (Sigma) diluted in TBS, and incubated for 30minutes. After washing with TBS, Alkaline Phosphatase buffer. The Alkaline phosphatase buffer (see Appendix 1) made fresh each time, and filtered before use After 15-20 minutes, the substrate was removed by washing for 2 minutes with water. Sections were counterstained in Haematoxylin for 2 minutes, and then washed in water for 20 minutes. Slides were mounted using Aquamount, blinded and counted under a microscope. Each section had 5 fields of view counted for positive cells, 3 sections counted per mouse, and 3 mice counted per time point.

2.6.2 Immunohistochemistry of NALT

The heads from experimental and control mice were removed following exsanguinations under terminal anaesthesia and fixed for at least 24 hours in 10% formalin (Sigma, UK). After rinsing in EDTA decalcification solution (0.43M EDTA, pH 7), the head was trimmed to leave the nasal passages. The heads were re-immersed in EDTA decalcification solution at 37°C for at least 7 days, rotating slowly. The incisors were removed, and the heads were rinsed in H_2O, placed nose down into deep cassettes for paraffin embedding, and 10μm sections were cut from -20°C chilled paraffin blocks (to obtain good sections) onto in situ PCR Glass slides (Perkin Elmer). Slides were dried overnight at room temperature, and de-waxed by successive immersion in 2 changes in xylene (Sigma), (10 minutes each), 2 changes in 100% ethanol followed by 2-3 rinses in distilled water and a final immersion in 10mM Citrate buffer pH6, heated in a microwave for 5 minutes. Slides were cooled for 2-3 minutes, washed 2-3 times in PBS and then immersed in acetone at –20C, for 10 minutes, followed by PBS.

2.6.3 Sections of Cervical Lymph Node.

Cervical lymph nodes (CLNs) from experimental and control mice were harvested from the neck, and stored on liquid nitrogen. In appearance they are small nodules of hardened tissue. Frozen CLNs were embedded in OCT (Electron Microscope Services), and 10μM sections were cut onto poly-L-lysine (Sigma, UK) coated in situ PCR slides (Perkin Elmer). Before staining, slides were air dried, fixed, for 10 minutes in acetone at –20°C, and then air dried. Sections were washed in PBS, blocked with 4% normal rabbit serum (Vector laboratories) for 1 hour then stained with rabbit anti-GFP FITC (Sigma, UK) at 1:50 for 1 hour. Sections were washed with 4 changes of PBS, dried, covered with prolon

aqueous mount, and a glass coverslip, and then dried overnight in the dark. Fluorescence was examined using an immunofluorescence microscope (Photonic sciences, Nikon).

Chapter 3 – Optimising plasmid DNA delivery with cytofectins

3.1 Introduction

DNA vaccines encoding the antigens of interest exhibit several beneficial properties as compared to alternative vaccine delivery systems (ie recombinant subunit or viral vectors). These include the ease of antigenic manipulation (via insertions/deletions/mutations of DNA coding sequences), relative stability of plasmid DNA and ease of manufacture. Despite these clear benefits, there remain obstacles to overcome. Although DNA vaccines demonstrate efficacy in several murine models of infectious disease, they lack potency in human and non-human primates. Lack of efficacy relates in part to degradation of naked DNA *in vivo* by endonucleases and in part inefficient cellular uptake and escape from the endosome (Lechardeur *et al.* 1999) and poor expression in non dividing cells (Escriou *et al.* 2001).

There are a number of barriers, both extra- and intracellular, that needs to be overcome before plasmid DNA can transfect cells *in vivo*. The degradation of naked plasmid DNA due to the presence of endonucleases within the extracellular milieu is the primary barrier to transfection. Once within the cell the plasmid DNA needs to escape from the endosome into the cytosol, and then from the cytosol into the nucleus before the plasmid can be transcribed (Figure 3.1.1).

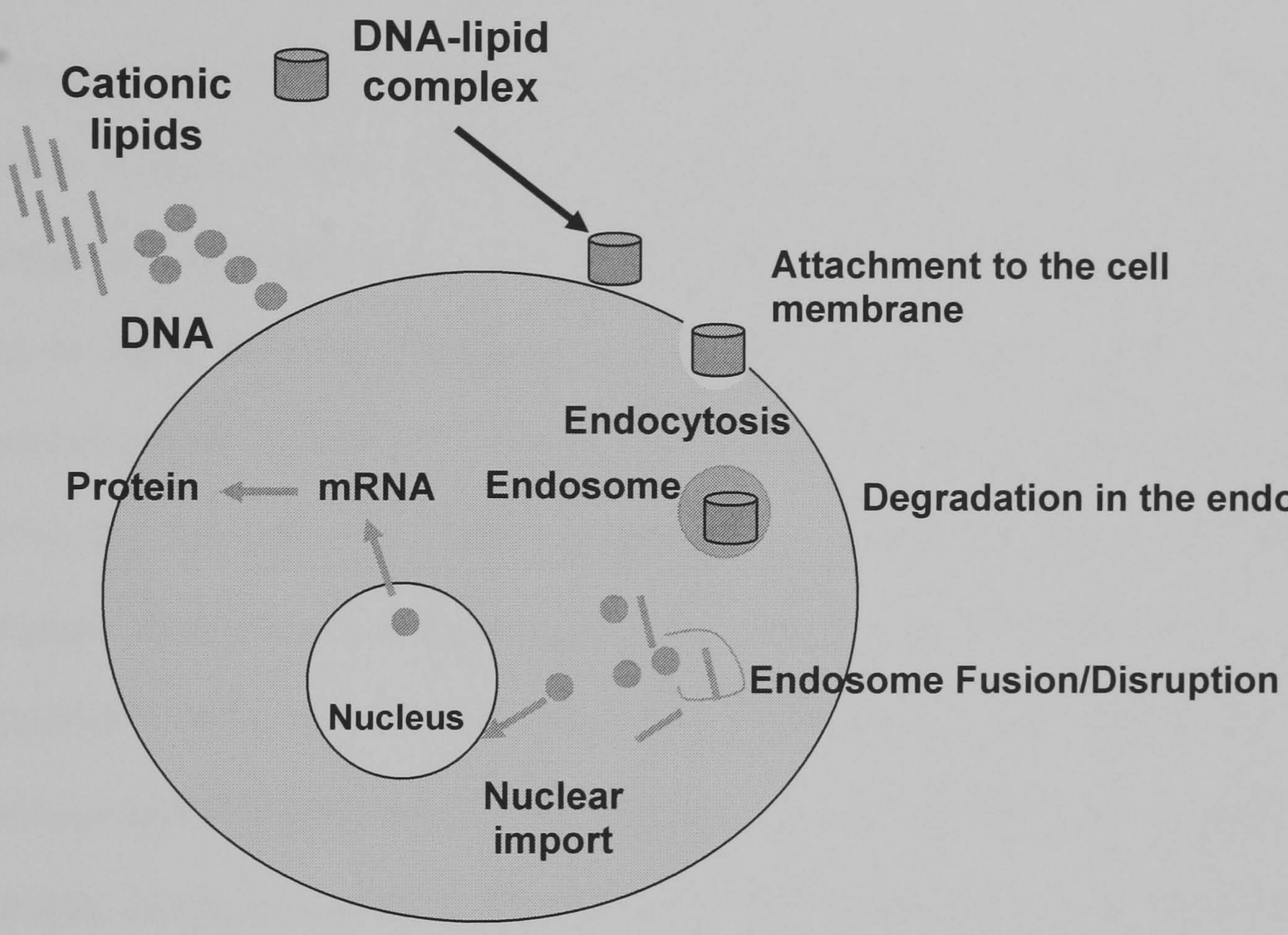


Figure 3.1.1, Cellular barriers in gene transfer

Although viral vectors are still superior in their transfection efficiency (MacLachlan *et al.* 1999), their application raises a number of safety concerns (Ilan *et al.* 1997; Rubanyi 2001). These include possible reversion of the viral vector to a pathogenic form or recombination with wild type viruses and hence propagation of recombinant DNA in the environment. Further potential problems with viral delivery systems include the induction of dominant immunological responses to the viral vector as opposed to the transgene, and pre-immunity to the vector which greatly reduce efficacy (Roth *et al.* 2002). Conversely plasmid DNA is replication defective, non-pathogenic and non-antigenic. Complexes of DNA and cationic lipids, termed lipoplexes, have been shown to act as highly effective alternatives for free DNA (Felgner *et al.* 1994). The rationale behind cationic lipids is their ability to facilitate transfer of the heavily charged plasmid DNA into the cytosol. The lipids used to complex plasmid DNA consist of a positively charged lipid, these include dimyristyloxypropyl-3-dimethyl-hydroxyethyl ammonium (DMRIE) (San *et al.* 1993; Wheeler *et al.* 1996; Klavinskis *et al.* 1997) and (+/-)-N-(3-aminopropyl)-N,N-dimethyl-2,3-bis (dodecyloxy)-1-propanaminium bromide (GAP-DLRIE) (Stephan *et al.* 1996; Wheeler *et al.* 1996; Sankar *et al.* 2002) which bind to the negative charge of the phosphate plasmid DNA backbone. These are combined with a neutral lipid to allow fusion to the plasma membrane and escape from the endosome to the cytosol.

It is believed that transfection activity increases when the chain length and degree of saturation of the hydrophobic alkyl or acyl chains decreases (Felgner *et al.* 1994). These changes induce an increase in the fluidity of the cationic lipids. It is this fluidity, which

not only determines the final size of the complexes (Akao *et al.* 1996; Regelin *et al.* 2000), but may also act to destabilise the cellular membranes, thus increasing the transfection efficiency (Koltover *et al.* 1998; Hirsch-Lerner and Barenholz 1999).

Another key component in the formulation of the lipid is the inclusion of a helper lipid. The addition of this helper lipid can alter the fluidity of the complexes. When DNA is added to the lipid bilayers the relative fluidity is reduced (Regelin *et al.* 2000), but if the formulation is a mix of cationic/zwitterionic lipids, then the binding of DNA to the lipid only induces minor changes in the fluidity of the lipid. (Hirsch-Lerner and Barenholz 1999; Regelin *et al.* 2000). Many studies have confirmed that higher transfection levels can be observed in lipoplexes containing cationic lipids with co-lipid combinations (Felgner *et al.* 1994; Balasubramaniam *et al.* 1996; Ferrari *et al.* 2001; Heyes *et al.* 2002). However, the addition of a zwitterionic lipid remains contentious since there has been a reported an induction of complex rigidity due to the inclusion of curtain zwitterionic lipids (Zuhorn and Hoekstra 2002), thus careful consideration of the helper lipid used in the complex is required. An example of a helper lipid is dioleoyl-phosphatidyethanolamine (DOPE), this is a zwitterionic lipid that can help maintain the fluidity of the DNA bound lipid complex (Regelin *et al.* 2000), and stabilise the cationic lipid suspension, as cationic lipids repel each other (Zuidam and Barenholz 1998). When DOPE is complexed with DMRIE it has been reported to improve transfection efficiencies and allows increased doses of DNA to be delivered *in vivo* (San *et al.* 1993).

An important factor in transfection efficiency is the environment in which the plasmid DNA complexes with the cationic lipids. There are many factors which can effect the complexing; the size of the plasmid DNA and cationic lipid complex, the concentration of lipid and plasmid DNA at the time of mixing, the mixing rate, order of addition, ionic strength of the mixing buffer, and the cationic lipid/DNA charge ratio have all been shown to represent a minimal set of parameters that affects lipoplex characteristics. However, when the same reagents and same concentrations, but different formulation procedures are used the resulting thermodynamic and biological stability of lipoplexes may again differ greatly (Thierry *et al.* 1997; Ferrari *et al.* 2001). Transfections *in vitro* also present multiple variables; the transfection competence of the cell type used (Son *et al.* 2000), cell seeding density (Lascombe *et al.* 1996), the degree of transformation of the cells (Harrison *et al.* 1995) and the stage of cellular replication cycle (Pickering *et al.* 1994). Culture condition related effects include the presence of serum in the transfection mixture (Hwang *et al.* 2001), dilution of lipoplex suspension (Staggs *et al.* 1996), the time lipoplexes are incubated with the cells (Zabner *et al.* 1995), type and concentration of salts and other biomolecules present in the liposome-nucleic acid mixing medium (Wasan *et al.* 1999).

Upon contact with the cell, the lipoplex is taken up through endocytosis, however, the actual binding to the cell membrane, has still to be elucidated. Reports indicate that clathrin-mediated endocytosis is required. This has been indicated by the observation that transfection is inhibited by cyclodextrin induced cholesterol removal and resolved upon cholesterol resupply. (Zuhorn *et al.* 2002). Others have shown that a fusion event,

between the membrane and the lipid is required to induce endocytosis (Almofti *et al.* 2003). However, though the majority of reports suggest that lipoplex uptake occurs via an endocytosis mediated pathway, there are also some reports that the lipoplexes can directly fuse with the membrane and hence deliver DNA directly into the cytoplasm (Felgner *et al.* 1987; Pires *et al.* 1999).

Once the plasmid has entered the cell, the next barrier is to escape the endosome. It is proposed at this stage, a fusion between the endosomal membrane and the lipoplex takes place, causing the DNA to be dissociated from the lipid and delivered to the cytosol. (Nakanishi and Noguchi 2001). The co-lipid DOPE has been attributed with the properties to facilitate membrane fusion and aid in the destabilisation of the plasmalemma or endosome (Felgner *et al.* 1994).

The final cellular barrier is the translocation of the plasmid across the nuclear pore. The exact method of uptake into the nucleus is, as yet, unknown. However, certain criteria are documented in other fields of study. For example, the import of large nucleic acids requires the association with proteins. This is potentially similar to the transport of adenoviral genomic DNA, which binds endogenous histones to be transported into the nucleus. (Trotman *et al.* 2001). Another hypothesis is that mitosis is required for plasmid DNA to gain access into the nucleus, based on the observation that there is a close correlation between the onset of transgene expression and mitosis in synchronised cell cultures. (Brunner *et al.* 2000; Escriou *et al.* 2001). Nuclear import may play an important role as the half-life of plasmid DNA in the cytosol is recorded to be 90 minutes

(Lechardeur *et al.* 1999). However, cell division is essential for efficient transgene expression (Escriou *et al.* 2001). Cells that have been arrested in the G1 phase of the cell cycle with aphidicolin only poorly express micro injected DNA. Furthermore, only the cells that escaped G1 arrest expressed the plasmid DNA efficiently (Mortimer *et al.* 1999; Escriou *et al.* 2001).

Lipid cytotoxicity is an important consideration in the design of a DNA-lipoplex based vaccine strategy. In cell culture, lipoplexes cause changes to cells, including cell shrinking, reduced number of mitotic events, and vacuolization of the cytoplasm. (Lappalainen *et al.* 1994). The effects *in vivo* of cationic lipids at certain concentrations are likely to be equally as toxic. Positively charged lipoplexes have been noted to activate complement via the alternative pathway (Chonn *et al.* 1991). Intratrachael administration of lipoplexes has shown that a significant quantity of plasmid DNA enters the nuclei after release from lipoplexes. These cells undergo cell death, and thus result in low levels of transgene expression. (Uyechi *et al.* 2001). While lipoplex associated cytotoxicity may be detrimental to the levels of transgene expression, necrosis or apoptosis of transfected cells may actually enhance the immune response to the transgene. Cell death is associated with induction of inflammatory responses and hence recruitment of DCs required to prime an immune response to the expressed transgene.

In this current study, we examined the efficiency of plasmid DNA transfection of cell lines. We investigated the relative efficiency of *in vitro* transfection of multiple cell lines with plasmid DNA constructs, at different nucleotide:cytofectin molar ratios. An

optimised transfection protocol was derived from this *in vitro* study and applied to an *in vivo* investigation. The efficiency of plasmid DNA lipoplex transfection was evaluated when delivered via the intra-nasal route and the levels of transgene expression examined at both the cellular and tissue level.

3.2 Results

3.2.1 Increasing the Molar ratio of Cytofectin:nucleotide, increases the transfection efficiency of 293T cells.

Cytofectins have been widely used to increase the efficiency of plasmid DNA transfection. A number of properties can be investigated to improve the transfection efficiency generated by the complexing of plasmid DNA to the cytofectins. The properties investigated were the effects of plasmid DNA to cytofectin ratio on the efficiency of transfection, by quantifying the production of the plasmid encoded reporter gene product. The lipids GAP-DMORIE:DpyPE and DMRIE:DOPE were complexed with plasmid DNA encoding the reporter gene Secreted Alkaline Phosphatase (SEAP), at a range of molar ratios (nucleotide:cytofectin). Plasmid DNA complexed with the cytofectin was added to HEK293T cells for 48 hours, and the supernatants were tested for the presence of SEAP. The results show a dose dependant increase in SEAP production (hence transfection efficiency) peaking at 0.25:1 and 0.5:1 (Figure 3.2.1a). Similar results were observed using the intracellular reporter gene, Green Fluorescent protein (GFP) (Figure 3.2.1b). However, a dip in transfection was observed at the highest ratio of cytofectin (0.5:1). This result implies a toxicity at high cytofectin concentrations, which was not apparent using SEAP as a reporter system.

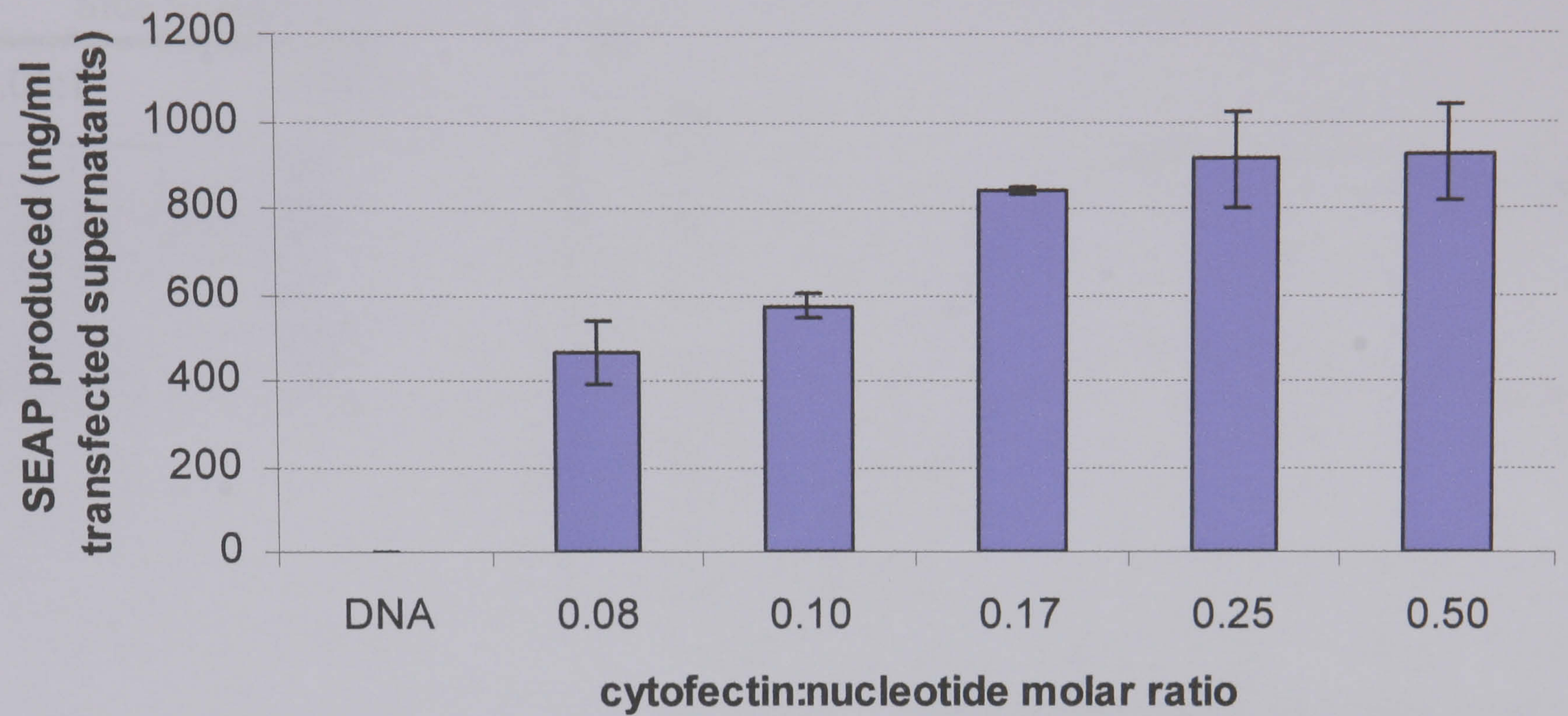
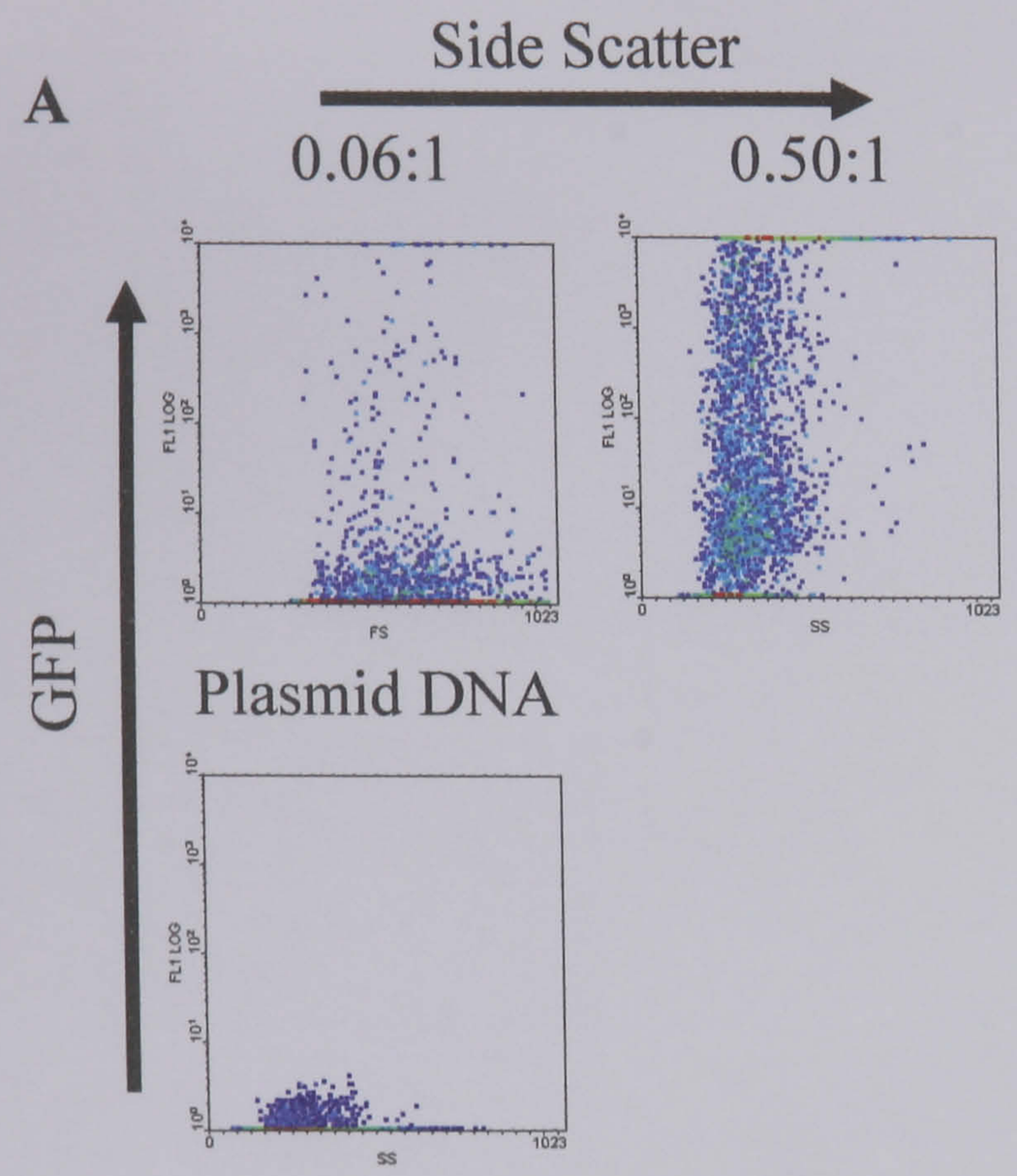


Figure 3.2.1a, Increasing the Molar ratio of cytofectin:nucleotide (DNA), increases the transfection efficiency of HEK293T cells with a plasmid encoding SEAP. Plasmid DNA encoding SEAP was complexed with the cytofectin DMRIE:DOPE at the cytofectin:nucleotide (DNA) molar ratios of 0.50:1, 0.25:1, 0.17:1, 0.13:1, 0.1:1, 0.08:1and then added to HEK293T cells. After 48 hours, supernatants were harvested and assayed for SEAP expression. The concentration of SEAP produced was determined from a standard curve and the results represent the mean and standard deviation from 8 replicates.



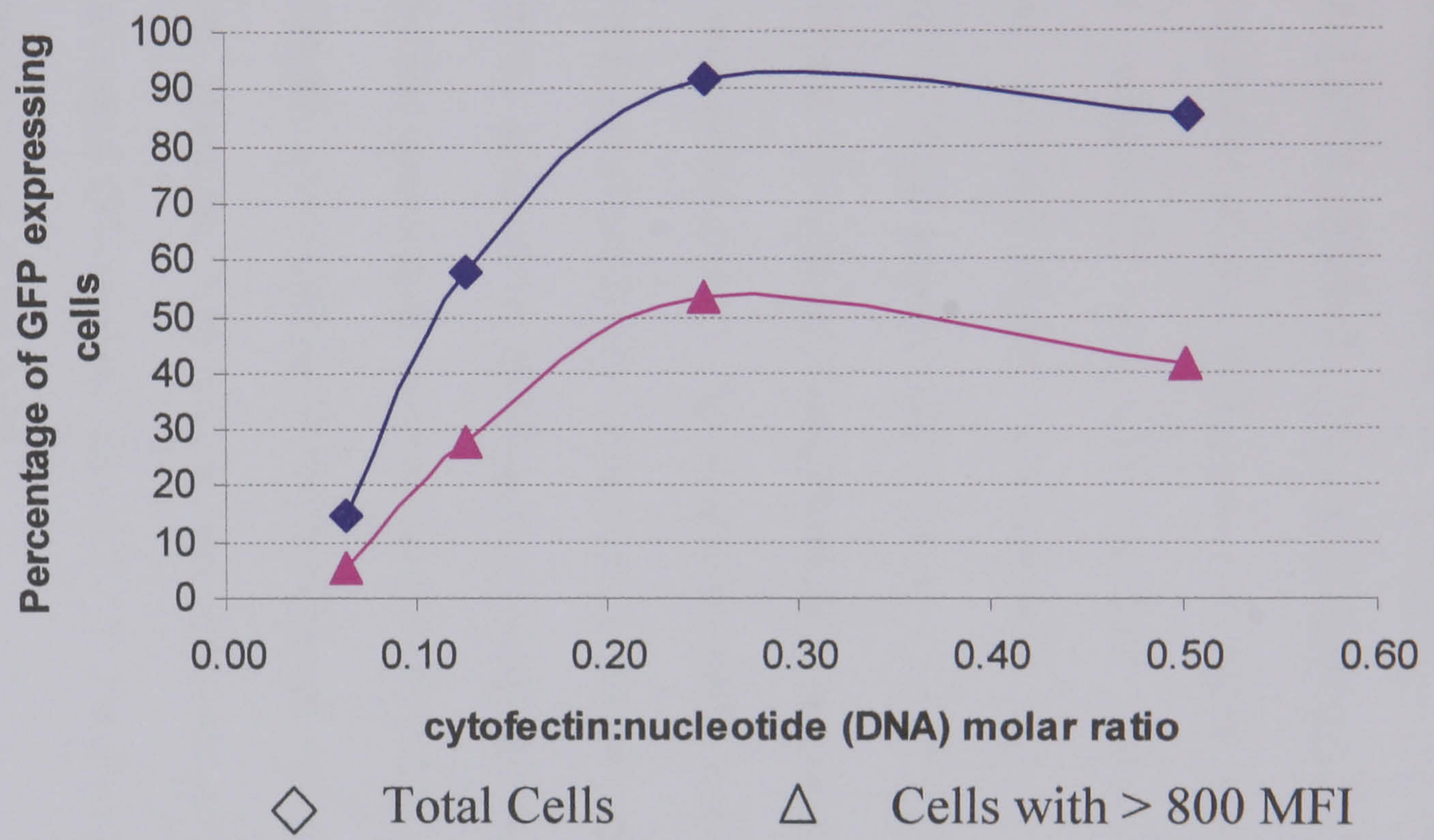


Figure 3.2.1b, Increasing the Molar ratio of Cytofectin:nucleotide (DNA), increases the transfection efficiency of HEK293T cells with a plasmid encoding GFP. Plasmid DNA encoding GFP was complexed with the cytofectin DMRIE:DOPE at the cytofectin:nucleotide (DNA) molar ratios of 0.50:1, 0.25:1, 0.13:1, 0.06:1and then added to HEK293T cells. After 48 hours, cells were harvested and assayed for GFP expression. A) Examples of flow cytometric plots at the different cytofectin:nucleotide DNA ratio. B) Total cells expressing GFP (◊) and cells expressing GFP with a mean fluorescent intensity greater than 800 (Δ).

3.2.2 By changing the lipid that complexes the DNA, the *in vitro* transfection efficiencies of 293T cells with a plasmid encoding SEAP, can be altered.

Although a number of lipid formulations could be potentially used for lipoplex based transfection, efficiencies vary with the lipid species used. Three proprietary lipid formulations (DMRIE:DOPE, GAP-DMORIE:DpyPE and GAP-DLRIE) under development by Vical were available for *in vivo* evaluation. It was therefore important to determine which of the three formulations had the greatest transfection potential *in vitro* prior to *in vivo* investigation. This was achieved by complexing a plasmid encoding the reporter SEAP, with each of the cytofectins at a cytofectin:nucleotides (DNA) molar ratio of 0.25:1, 0.13:1 and 0.06:1. The lipoplexes were then transfected into HEK293 cells for 48 hours and the supernatants assayed for SEAP activity. At a ratio of 0.25:1 both GAP-DMORIE:DpyPE and DMRIE:DOPE transfected the cells at a higher efficiency than GAP-DLRIE. However, GAP-DLRIE was efficient at the lower concentrations and as compared to naked DNA alone (Figure 3.2.2). On the basis of this result, GAP-DLRIE was then eliminated from this study.

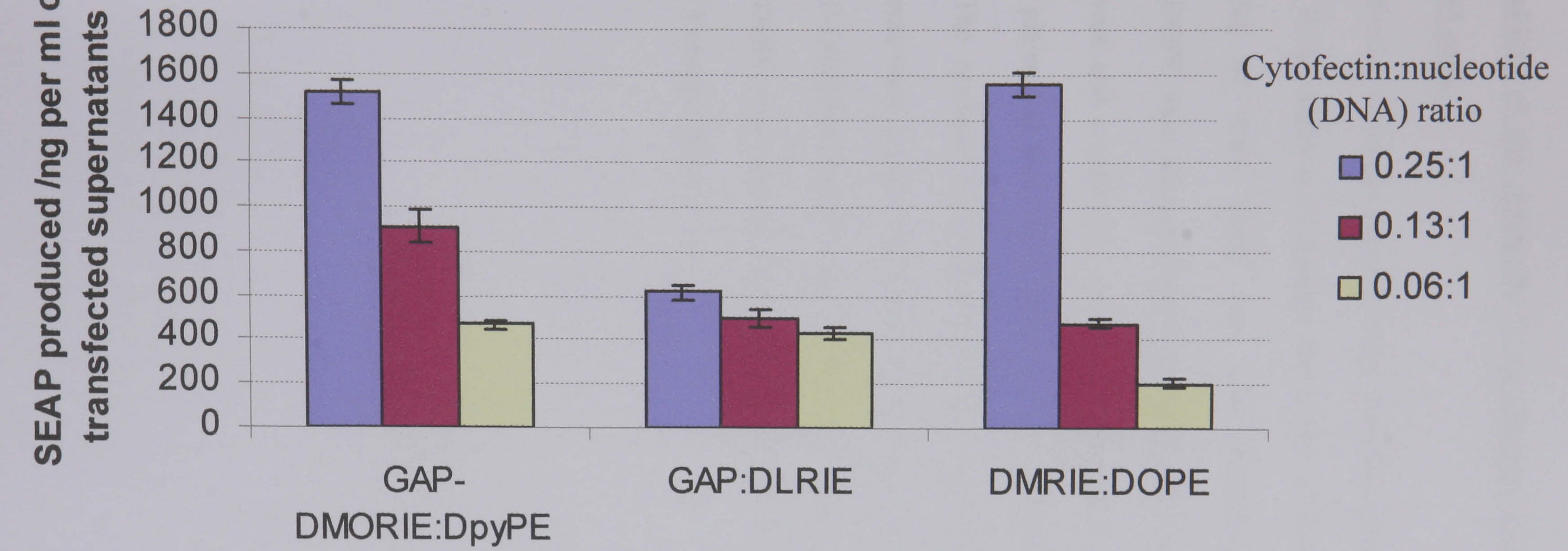


Figure 3.2.2, By changing the cytofectin that complexes the DNA, the *in vitro* transfection efficiencies of 293T cells with a plasmid encoding SEAP, can be altered. Plasmid DNA encoding SEAP was complexed to either GAP-DMORIE:DpyPE , DMRIE:DOPE, or GAP:DLRIE, at the cytofectin:nucleotide (DNA) molar ratios of 0.25:1, 0.13:1 and 0.06:1 and added to HEK293T cells. After 48 hours, supernatants were harvested, and tested for SEAP activity. A standard curve using recombinant SEAP was generated to calculate the amount of SEAP produced, and results represent the means and standard deviations from 5 replicate transfections.

3.2.3 Stability of the cytofectin is paramount to the efficiency of transfection of HEK293T cells.

There are many issues to consider when choosing cytofectins for a vaccine strategy, one of these is the stability of storage. The effect of freeze-thawing upon the transfection efficiencies of these lipids was also investigated. GAP-DMORIE:DpyPE and DMRIE:DOPE were aliquoted and half the aliquots frozen at –20°C. The aliquots were then thawed and complexed, along with non-freeze thawed aliquots, with the SEAP encoded plasmid at the molar ratios of 0.25:1, 0.13:1 and 0.06:1 (cytofectin:nucleotide DNA). The resultant lipoplexes were then transfected into HEK293 cells and the supernatants were assayed for SEAP activity after 48 hours of culture. The results show that GAP-DMORIE:DpyPE was unaffected by the freeze-thaw but the efficiency of DMRIE:DOPE was significantly reduced at the 4:1 ratio ($p=0.0004$ using a paired Students T-test) (Figure 3.2.3).

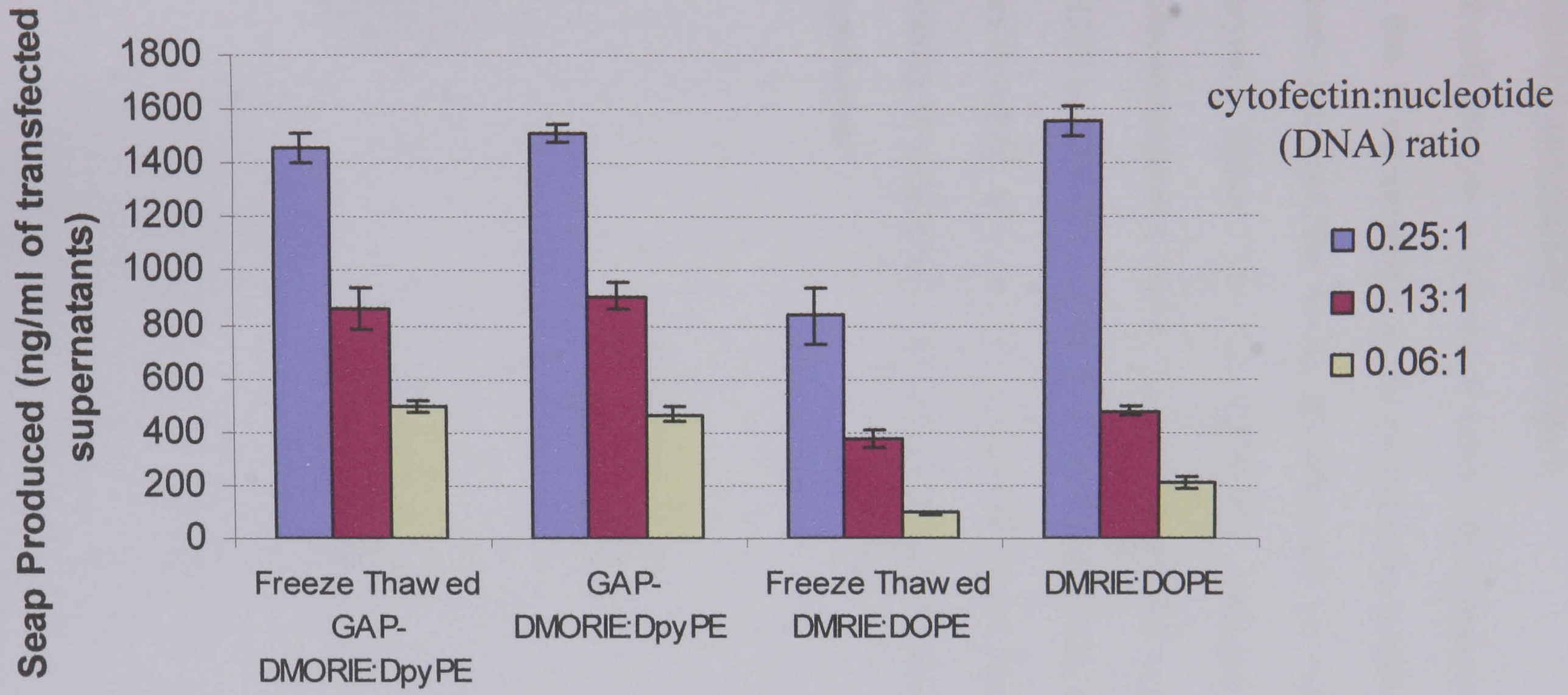


Figure 3.2.3, The stability of cytofectins upon freeze thawing can effect the transfection efficiency of a transgene. HEK293T cells were transfected with a plasmid expressing SEAP complexed to GAP-DMORIE:DpyPE , DMRIE:DOPE, and aliquots of both DMRIE:DOPE and GAP-DMORIE:DpyPE had undergone freeze thawing, at the cytofectin:nucleotide molar ratios of 0.25:1, 0.13:1 and 0.06:1. After 48 hours, supernatants were harvested and tested for SEAP activity. A standard curve was generated to calculate the amount of SEAP produced in the culture and the results represent the mean and standard deviation from 5 replicate transfections.

3.2.4 The effect of Cytofectin:nucleotide ratio on the transient transfection efficiency in NIH3T3 and HEK293T cell lines.

Each cell line has a different potential for transfection, therefore just investigating one cell line is unrepresentative. To examine the transfection efficiency of DMRIE:DOPE between cell lines, the murine fibroblast cell line NIH3T3 was compared to the human embryonic kidney cell line HEK293. Plasmid DNA was complexed at the cytofectin:nucleotide (DNA) molar ratios of 0.5:1 and 0.17:1 and added to either the NIH3T3 or HEK293T cells. The supernatants were assayed for SEAP activity after 48 hours in culture and clearly indicated that the cell line NIH3T3 was transfected at a low efficiency as compared to the HEK293T cells (Figure 3.2.4). This result was repeated in 3 experiments.

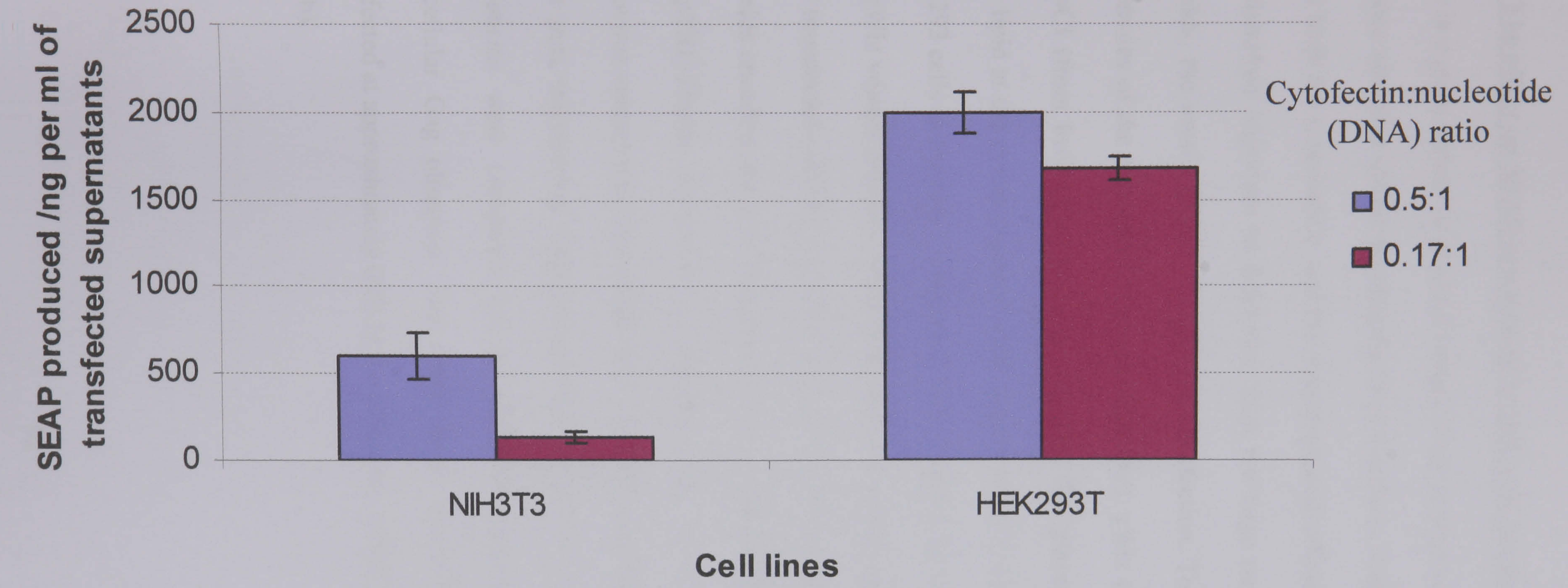
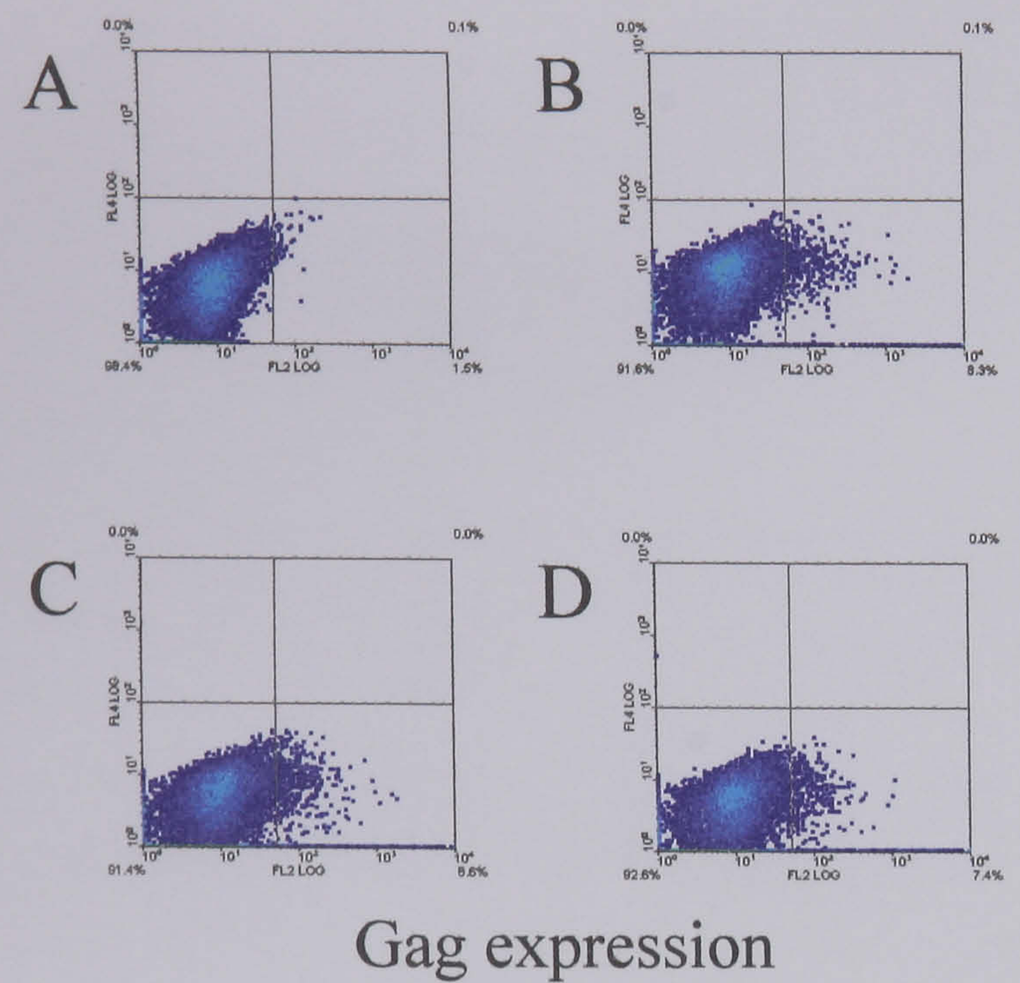


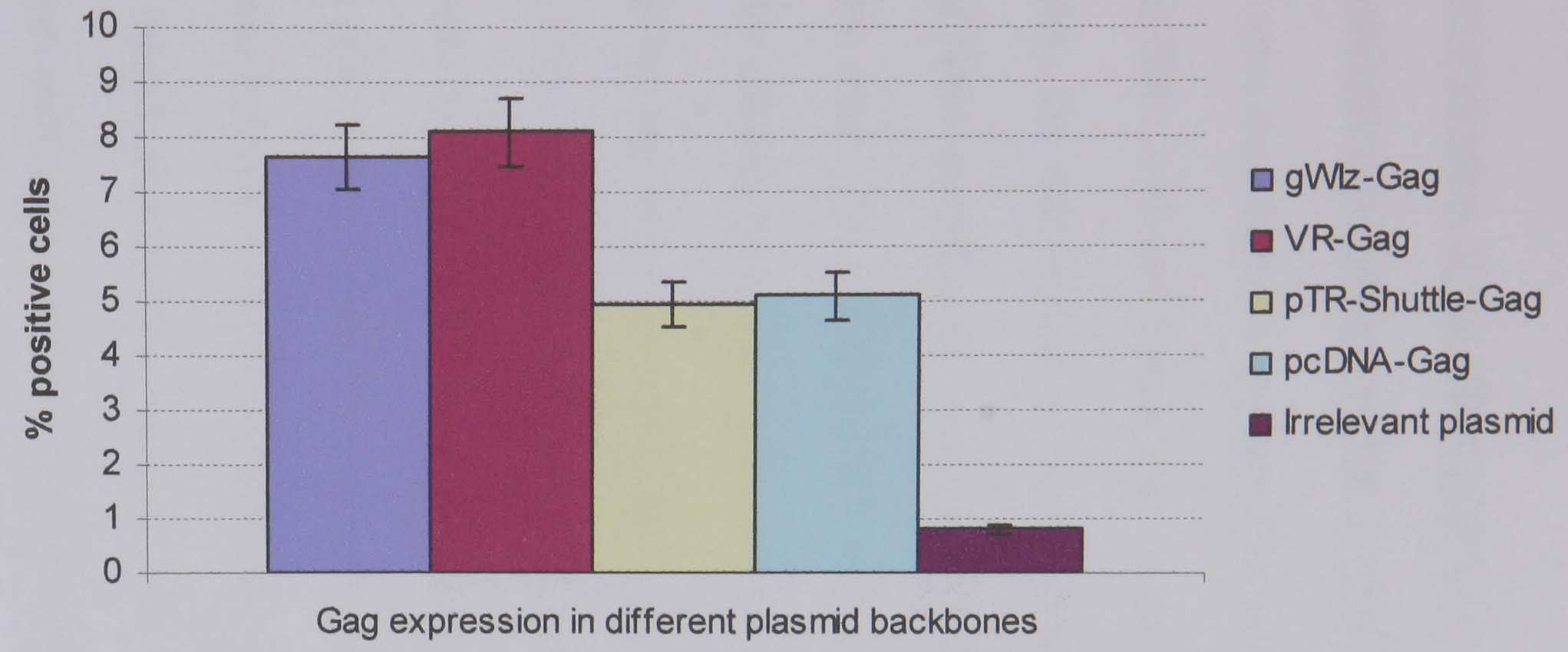
Figure 3.2.4, The efficiency of lipoplex transfection in cell type specific; with the optimal ratio of cytofectin to nucleotide varying with different cell types. HEK293T cells or NIH3T3 cells were transfected with DMRIE:DOPE at the ratios of 0.5:1 and 0.17:1. After 48 hours the supernatants were harvested, and tested for SEAP activity. A standard curve was generated to calculate the amount of SEAP produced in the culture.

3.2.5 The affect on SEAP production by different plasmid constructs.

There is a great variety in plasmid design, from differences in the promoter sequence, to sequence variation within the plasmid DNA backbone. Changes in the basic plasmid may affect both the transfection and the transcriptional efficiencies of the plasmid DNA. It was therefore important to determine from the large range of commercially available plasmids, the most efficient plasmid for transfection. To achieve this, the transfection efficiencies of the plasmids VR (from Vical Inc), gWiz (from Gene Therapy Systems), and pCI (from Invitrogen) were investigated. The plasmids were complexed with the same lipid at the cytofectin:nucleotide (DNA) molar ratio of 0.25:1 and transfected into HEK293 cells as previously described. The resultant SEAP production show that the VR and gWiz vectors transfect HEK293T cells to a comparable level, which is greater than those transfected with the pCI vector (Figure 3.2.5). Similar results were also obtained for plasmids encoding the HIV Gag protein. The plasmids VR, gWiz, pcDNA (Invitrogen), and pTRE-Shuttle (Clontech - a plasmid used in the generation of a recombinant adenovirus vector) were transfected into HEK293 cells as previously described. After 48 hours post transfection, Gag protein was stained intracellularly, and the transfection efficiencies were compared for the different plasmids based on the amount of intracellular Gag observed. The result shows pTRE-Shuttle and pcDNA plasmids transfected at approximately 60% of the efficiency of the gWiz and VR plasmids. (Figure 3.2.5b).

Figure 3.2.5a, The level of reporter gene expression is influenced by the plasmid DNA backbone. The vectors PCI, gWiz and VR encoding SEAP were used to transfect HEK293 cells at a cytofectin:nucleotide (DNA) molar ratio of 0.25:1. After 48 hours post transfection, supernatants were harvested and examined for the production of SEAP. A standard curve was generated to quantify the amount of SEAP produced. Values are the means of 4 replicates, with error bars being represented as the standard deviation between the replicates.





Gag expression

Figure 3.2.5b, The level of reporter gene expression is influenced by the plasmid DNA backbone. The vectors VR, gWIz, pTRE-Shuttle (an adenoviral shuttle vector) and pcDNA encoding HIV Gag was used to transfect HEK293 cells at a cytofectin:nucleotide (DNA) molar ratio of 0.25:1. After 72 hours, the intracellular HIV Gag was examined by flow cytometry. Panel A-D show examples of cells that are positive for Gag expression within HEK293 cells. Panel A results from transfection with an irrelevant plasmid, and B-D show the gag expression after transfection with the gWiz-Gag. Means were calculated from the number of Gag expressing cells from 5 replicate transfections, with the errors represented as the standard deviations between the samples (E).

3.2.6 Investigation of the effect of DNA dose and relative DNA to cytofectin ratios on the transfection efficiency within the NALT following intranasal inoculation.

The *in vitro* data that was generated in this study, defined the complexing conditions of plasmid DNA with cytofectins that optimised the transfection of cell lines. Using these principles, the transfection of NALT with plasmid DNA was investigated. The VR plasmid DNA encoding the reporter gene firefly luciferase was inoculated into the nares of balb/c mice. The plasmid DNA was complexed with the cytofectin DMRIE:DOPE either by altering the molar ratios (0.25:1, 0.13:1 or 0.06:1 cytofectin:nucleotide) or by altering the amount of plasmid DNA present (40μg, 60μg or 80μg) at the molar:molar ratio 0.13:1. After 72 hours the NALTs were extracted and examined for the presence of luciferase. The results show that at 40μg, no transfection was observed in any of the complexed ratios. However, when the range of plasmid DNA concentrations were examined luciferase was found maximally at the 60μg dose, with a reduction observed at 80μg. Transfection at 40ug was negligible. Each group was the mean of 4 mice, with the error bars represented as the standard deviation (Figure 3.2.6).

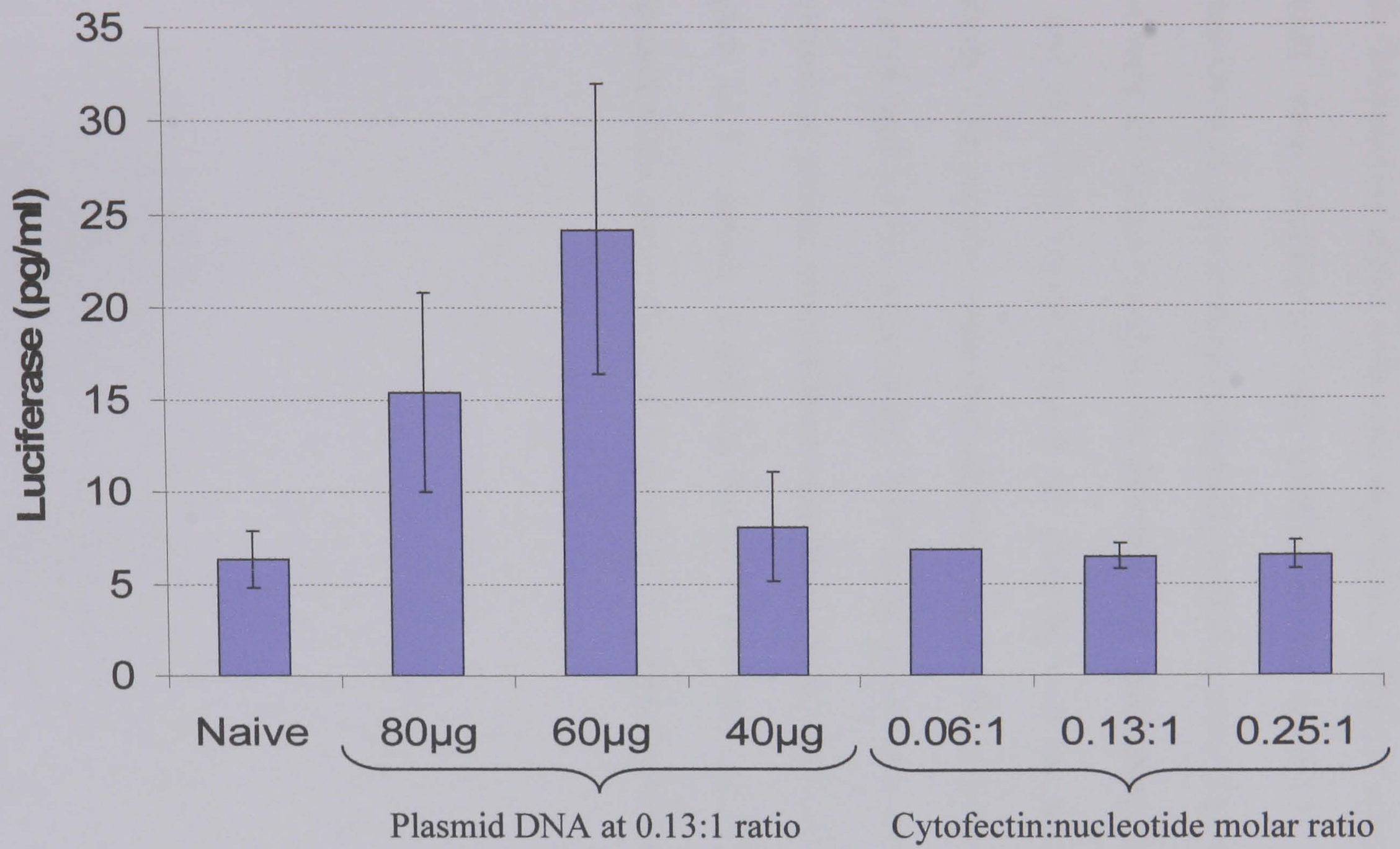


Figure 3.2.6, Transfection efficiency can be optimised *in vivo* by changing the amount of plasmid DNA, and the complexing ratio. 40µg of plasmid DNA was complexed with DMRIE:DOPE at the cytodectin:nucleotide molar ratios of 0.25:1, 0.13:1 or 0.06:1, or 40µg, 60µg or 80µg was complexed at 0.13:1, and inoculated intranasally into Balb/c mice. After 72 hours, the NALTs were harvested, and cellular contents were extracted and tested for luciferase content. Results represent the mean and standard deviation from 4 mice.

3.2.7 Intranasal immunisation with a DMRIE:DOPE complexed GFP expressing plasmid, shows GFP expression within NALT 48 hours post immunisation.

The previous experiment established that NALT tissue can be transfected with plasmid DNA. To examine which cells were transfected, 100µg of gWiz encoding GFP or control plasmid were complexed with DMRIE:DOPE at 0.17:1 molar ratio (cytofectin: nucleotide) and administered intranasally to Balb/c mice. The nasal area of the inoculated mice were dissected, fixed in 4% formalin and decalcified. Paraffin sections were then cut and the GFP signal boosted via staining with an anti-GFP (FITC) poly-clonal antibody. The sections were then stained with the fluorescent DNA collating dye DAPI and examined by UV microscopy. GFP staining was observed in cells surrounding the seromucosus glands, derived from mice administered with the GFP expressing plasmid, (Figure 3.2.7 - panels A and C). No GFP was detected from sections derived from recipients of the empty plasmid (Figure 3.2.7 - panels B and D).

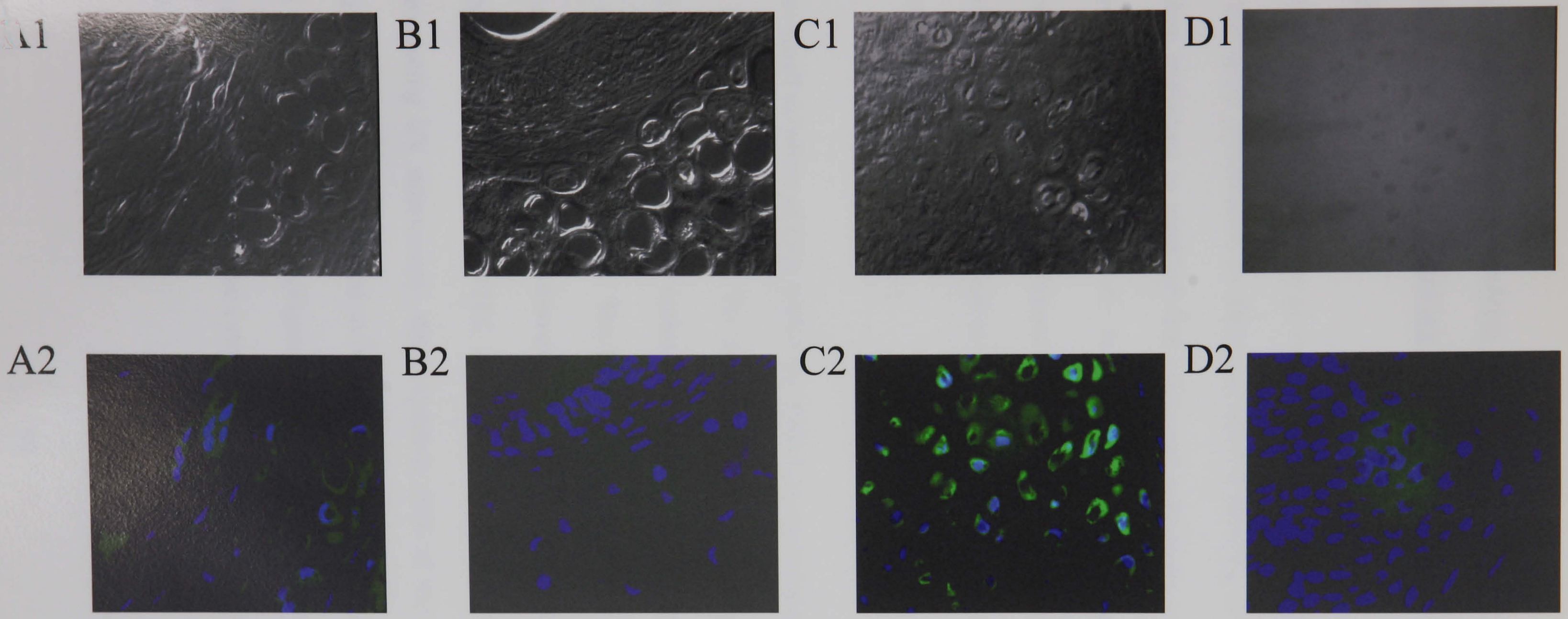


Figure 3.2.7, GFP is expressed within NALT 48 hours post immunisation. 100µg of a plasmid expressing GFP, gWiz-GFP (complexed with DMRIE:DOPE at the ratio of 0.17:1) was administered by the intranasal route to Balb/c mice. After 48 hours, the nasal cavities were harvested, and paraffin sections (transecting the nasal cavities) were prepared. GFP expression was visualized by amplification using an anti-GFP antibody, and counter stained with DAPI. A1-D1 are phase contrast views, A2-D2 fluorescent views at x40 magnification. A2 and C2 are representative regions of nasal; tissue demonstrating GFP expression from gWIz-GFP inoculated mice. B2 and D2, represent sections from mice receiving the vector.

3.2.8 Transgene positive dendritic cells can be detected locally and in the draining lymph nodes of mice following intranasal immunisation with GFP encoding lipoplex.

Although transgene expression *in vivo* is important to establish for an effective vaccine, only transgene presentation by DCs can achieve priming of the immune system. It was therefore important to establish that intranasal inoculation of the DNA resulted in DCs containing the transgene product. To address this point, 100μg of gWiz expressing GFP or control plasmid, was inoculated intranasally into mice as described in the previous experiment. Mice were sampled at 48 and 72 hours post inoculation, NALT and CLN were removed by dissection.

Due to small numbers of DCs within the NALT, it was impractical to examine sections of NALT for GFP positive DCs. To circumvent this problem, a single cell suspension was made from the pooled NALT within each group, and stained for the DC marker CD11c with a PE-Cy5 labelled mAb. The DCs were then sorted by flow cytometry and dried onto a glass slide to form a cytosmear. The cells were then acetone fixed, the GFP signal boosted with an anti-GFP FITC conjugated polyclonal antibody and examined by deconvoluting UV microscopy. Approximately 60% of the DCs sorted from the gWiz inoculated mice were observed to be GFP positive where as the DCs from the control mice were all GFP negative (Figure 3.2.8). The deconvaluted image clearly shows the GFP to be contained within the cytoplasm, whereas the CD11c staining only on the cell surface.

Frozen sections were taken of the CLN of the inoculated mice from both 48 and 72 hours. The sections were acetone fixed, and stained for CD11c and GFP as before and examined by UV microscopy. After 48 hours GFP positive DCs were not observed in either group (data not shown). However, by 72 hours GFP/CD11c double positive cells could clearly be seen in the mice inoculated with gWiz-GFP (Figure 3.2.8). These results clearly indicates the presence of transgene positive DCs both locally at the site of inoculation by 48 hours and at the draining lymph node by 72 hours.

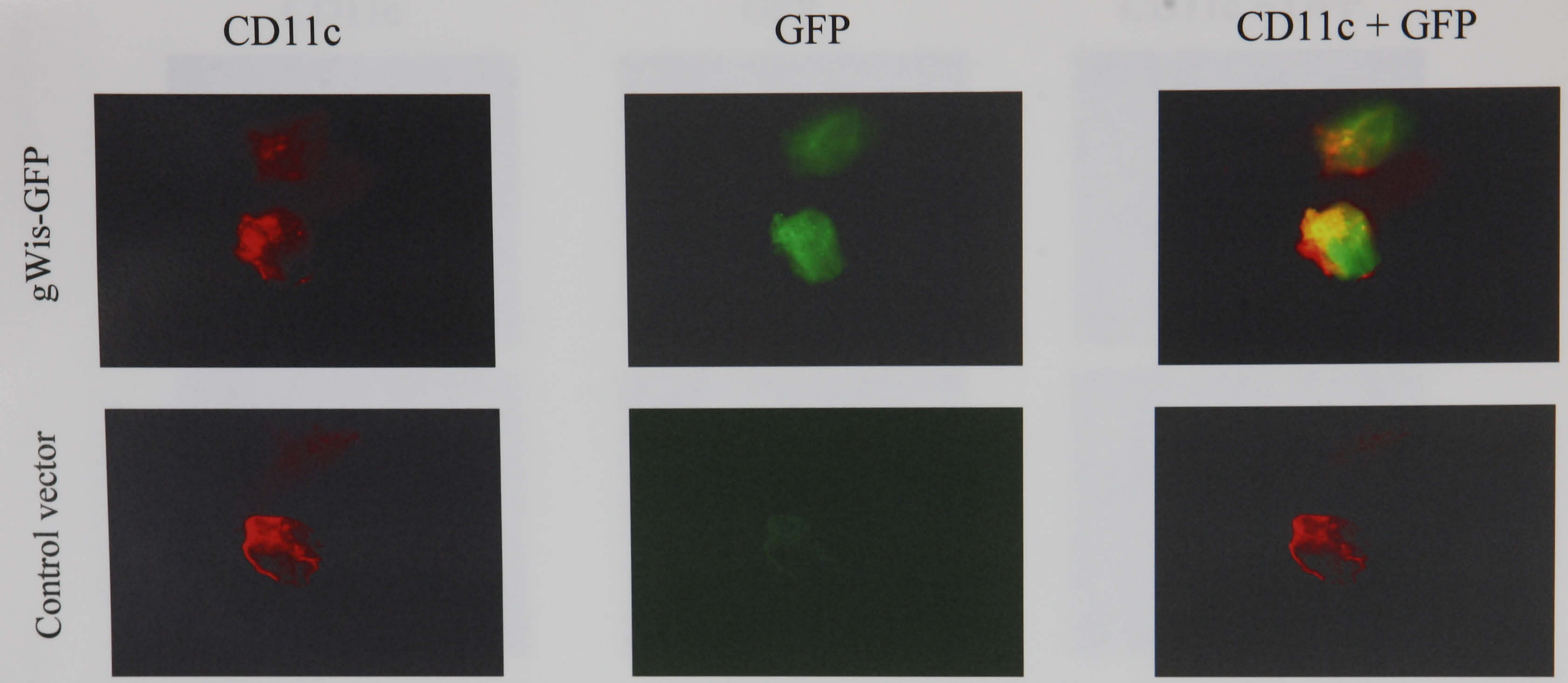


Figure 3.2.8, GFP is expressed within CD11c+ cells harvested from NALT 48 hours following intranasal inoculation of a lipoplex complexed plasmid encoding GFP. Mice were inoculated with 100μg of gWiz-GFP complexed at cytofectin:nucleotide molar ratio 0.17:1 with DMRIE:DOPE by the intranasal route. After 48 hours, the NALT was harvested, cells extracted, and the DCs were purified by flow cytometry based on their CD11c expression. GFP was visualised with an anti-GFP antibody. The panels show CD11c and GFP expression and also an overlay of CD11c with GFP for both the gWiz-GFP (top panels) and the empty vector control mice (bottom panels).

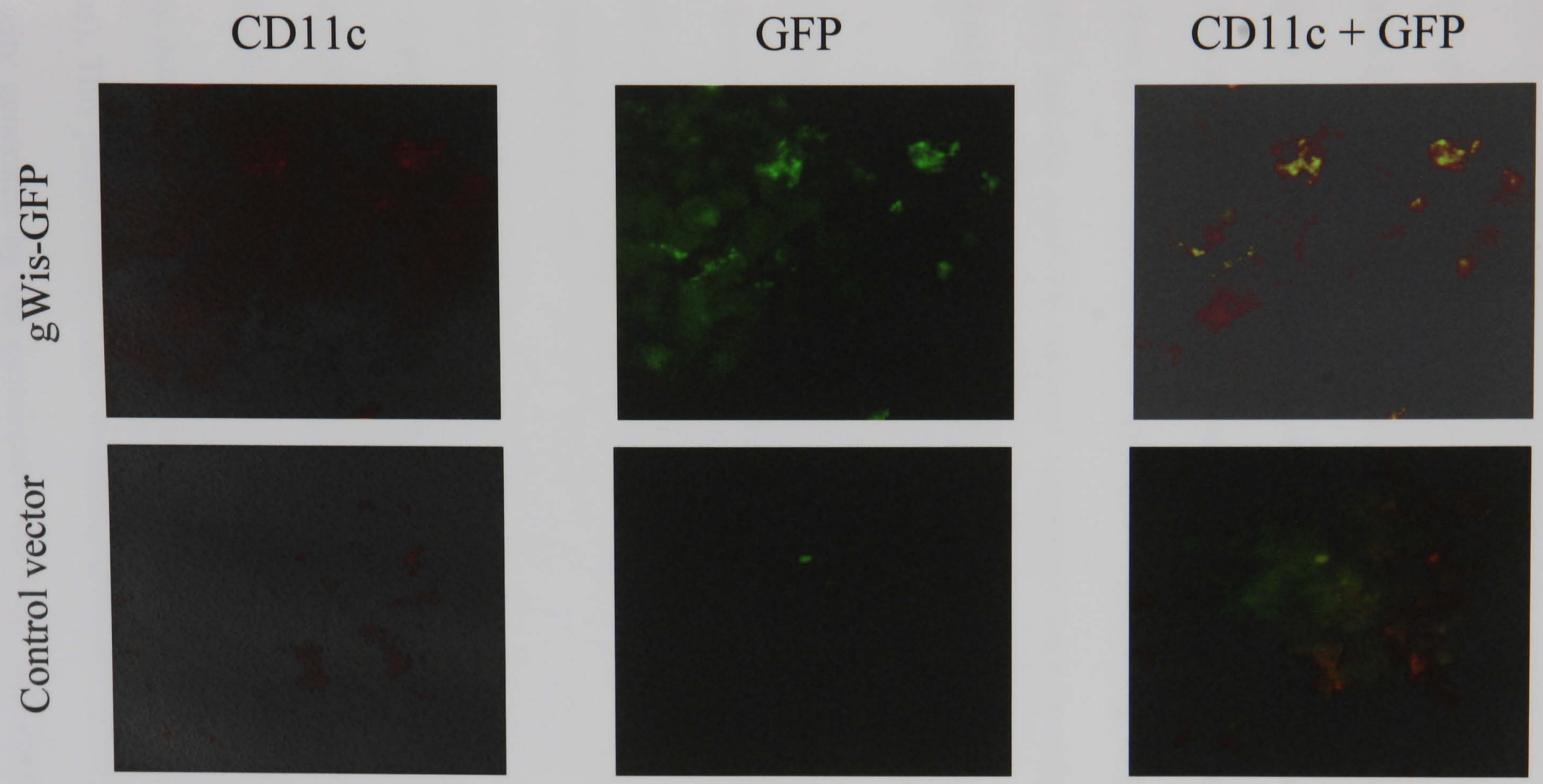


Figure 3.2.9, GFP is expressed within DCs in the CLN 48 hours following intranasal delivery of lipoplex complexed plasmid DNA encoding GFP. 48 hours following intranasal delivery of the plasmid gWiz-GFP complexed with DMRIE:DOPE (0.17:1 molar cytofectin:nucleotide (DNA) ratio), the cervical lymph nodes were harvested and frozen sections cut. GFP was visualised with an anti-GFP antibody, and counterstained with a monoclonal antibody to CD11c to identify DCs within the CLN. The panels show CD11c and GFP expression and also an overlay of CD11c with GFP for both the gWiz-GFP (top panels) and the empty vector control mice (bottom panels).

3.3 Discussion

For DNA vaccines to realise their full potential and translate to the clinic. will require the development of efficient delivery systems to enhance the level of plasmid encoded antigen produced. Possible approaches to overcome this hurdle include improving the plasmid by enhancing the promoter (Barnhart *et al.* 1998) or the use of cationic lipids (Felgner *et al.* 1994). This study examined the potential of improving the transfection efficiency of plasmid DNA by increasing the production of the transgene both *in vitro* and then *in vivo*. Conditions for cytofectin complexing of plasmid DNA were examined *in vitro* and the resulting optimal conditions were investigated *in vivo* to examine the level of transfection and which cells were transfected.

The study initially examined the effects of reducing the concentration of cytofectin upon the levels of transgene production within transfected cells. HEK293 cells that were transfected with a SEAP encoded plasmid, resulted in the accumulation of SEAP within the supernatants. When this plasmid was complexed with lowering concentration of the cytofectin DMRIE:DOPE, the amount of SEAP produced by the cells was decreased. The two highest concentrations of the cytofectin produced similar levels of SEAP (Figure 3.2.1a). However when intracellular reporter, GFP, and not a secreted reporter, SEAP, differences could be observed at the higher concentrations (Figure 3.2.1b), with less GFP being present at the 2:1 ratio than at the 4:1 ratio (molar:molar plasmid DNA:cytofectin ratios). This phenomenon could be explained by the toxicity generated by the cytofectin. Toxicity generated by the cytofectins has been well documented (Chonn *et al.* 1991; Lappalainen *et al.* 1994; Nita *et al.* 1996), and cell death upon transfection has also been

recorded (Uyechi *et al.* 2001). With SEAP being a secreted reporter, cell death of transfected cells will result in the SEAP being leaked into the supernatant, hence the amount of transgene product observed at the 2:1 ratio Cell death of the plasmid encoded GFP transfected cells results in the GFP being leaked away into the supernatant, reducing the number of GFP positive cells.

To examine the optimal transfection efficiency of plasmid DNA within a cell line, three cytofectins were investigated. The cytofectins examined where DMRIE:DOPE which is a 1:1 mixture of N-[1-(2,3-dimyristyloxy)propyl]-N,N-dimethyl-N-(2-hydroxyethyl)ammonium bromide (DMRIE) and dioleoyl phosphatidylethanolamine (DOPE), GAP:DLRIE which is ((±)-N-(3-Aminopropyl)-N,N-dimethyl-2,3-bis(dodecyloxy)-1-propanaminuim bromide) and GAP-DMORIE:DpyPE which is composed of (±)-N-(3-aminopropyl)-N,N-dimethyl-2,3-bis(cis-9-tetradecenyloxy)-1-propanaminium bromide (GAP-DMORIE), and the co-lipid 1,2-diphytanoyl-sn-glycero-3-phosphoethanolamine (DPyPE). The production of the transgene in cells transfected with the GAP:DLRIE complex plasmid DNA (Figure 3.2.2) was lower than those transfected with either the DMRIE:DOPE or the GAP-DMORIE:DpyPE. GAP:DLRIE is the only cytofectin tested that did not also contain a co-lipid, which for DMRIE:DOPE is DOPE, and for GAP-DMORIE:DpyPE is DPyPE. Similar to the results observed, the presence of a co-lipid has been documented to increase the transfection efficiency of plasmid DNA delivery (Felgner *et al.* 1994; Balasubramaniam *et al.* 1996; Ferrari *et al.* 2001; Heyes *et al.* 2002).

To examine if the conditions of the complexing of cytofectin to plasmid could be related to other cell lines, the cell line NIH3T3, a mouse fibroblast cell line, was transfected with SEAP encoded plasmid complexed with DMRIE:DOPE. Although transfection was observed the level of transgene production in NIH3T3 cells was reduced compared to the HEK293 cells (Figure 3.2.3). It has been reported that the composition of lipid bilayers may effect the uptake of the lipoplex (Ferrari *et al.* 2002), considering the difference between the lipid composition of a murine cell line and a human cell line, this could account for the differences in observed transfection efficiencies.

Another key component to plasmid transfection is the plasmid itself, a lot of commercial development has been geared towards increasing the efficiency of the CMV promoter, thereby increasing the duration and magnitude of the encoded antigen production. In trying to improve the efficiency of a plasmid vaccine, the right vector has to be chosen, and the efficiency of the CMV taken into account. By examining the transfection efficiencies of different plasmids, there is a clear distinction between the different plasmid backbones and promoters (figure 3.2.5a and figure 3.2.5b). However, the main point of contention here is that standardisation between preparations of DNA. If one plasmid preparation has a slightly lower optical density at 260nm versus 280nm, or higher LPS content, or hasn't completely dissolved homogeneously, the dynamics of cytofectin complexing can be altered and the transfection efficiencies effected. Work is taken to ensure that the DNA samples are of equivalent standard, with purification being done by Caesium chloride, the DNA ratios all correlating to around 1.8. and 80% of the plasmid being supercoiled.

Once the *in vitro* properties were investigated, the question became can these trends be translated into an *in vivo* situation. There are many considerations to be taken into account when going *in vivo*, especially when designing a vaccine strategy. The development of non-needle, patient compliant, vaccine strategies are being investigated for their applications in third world countries. These strategies include immunisation using the following routes, intranasal, oral, rectal, and with powder jet technology, intradermal. Previous publications, has shown the efficacy of intranasal immunisation in eliciting a humoral and cellular response (Gao *et al.* 1997), not only within the mucosa but also within the systemic compartment of the immune system (Klavinskis *et al.* 1997). In all *in vivo* work, strict guidelines are in place set by the Home Office, one of these guidelines limits the amount of inoculum that can be given intranasally. With a very limited dose, a delicate balance must be observed, between the amount of plasmid possible to fit into the volume, and the amount of lipid that can be complexed to it. As the concentration of the lipid and the plasmid increases, the amount of particulate precipitate increases, limiting cellular uptake thus transfection efficiency (Solodin *et al.* 1995). There are other problems with his method of immunisation, which is the dropwise addition of the inoculum onto the nares, resulting in the mouse sniffing the inoculum into its nasal passages and transfecting the nasal epithelium. If the application of the inoculum is too rapid, then the mouse either swallows it, or sneezes it out, making it difficult to regulate the exact amount that is absorbed by the nasal passages. The data shows a threshold of amount of plasmid is required for detectable levels of transfection (figure 3.2.8), which is not effected by the amount of lipid present. However, at a set ratio, the amount of plasmid that can be complexed with the lipid for an optimal transfection seems to cover a

limited range. The reasons for this could be purely mechanical. As discussed, at high lipid to DNA concentrations, the amount of precipitate increases, thus lowering the transfection efficiency. At the lower lipid concentrations, as all the volumes for immunisation were kept the same, the concentration of DNA being added per application, is insufficient to cause optimal transfection. After a limited number of applications of the inoculum to the nares, future applications become less effective as the nasal passages are coated. The optimal dosage is delivered in the first few doses. Thus requiring a balance between increasing the plasmid concentration, the lipid concentration, and the volume required for immunisation.

With the encoded antigen being found in the NALT 48 hours post immunisation (Figure 3.2.7), the ideal cellular target required for plasmid DNA is the DC. *In vitro* studies have highlighted the difficulties of transfecting DCs with plasmid DNA, with only a few reports claiming success (Yang *et al.* 1999; Denis-Mize *et al.* 2000). This leads to the question of why the DCs in the NALTs are so heavily bearing GFP expression. The answer is either the DCs are being directly transfected, which is unlikely due to the *in vitro* evidence, or that they are being cross-primed (Corr *et al.* 1999), and are taking up the GFP that has been released from dead or dying cells, or that was in cells the DCs have phagocytosed. This leads to an interesting question, if the DCs are being cross primed, by the contents of dead or dying cells, by increasing the toxicity of a vaccine, will you boost the efficacy of a vaccine? This certainly seems to be confirmed when co-immunisation with DNA vaccines which express Fas or a mutated version of a caspase gene which induce the creation of apoptotic bodies has been reported to enhance cell mediated

immunity (Chattergoon *et al.* 2000; Sasaki *et al.* 2001). Whatever the mechanism, the DCs do appear to contain the GFP with over 70% of total NALT DCs as determined by the CD11c expression containing GFP (Figure 3.2.9), and that cells expressing both GFP and CD11c can be found within the draining lymph node 48 hours post transfection (Figure 3.2.8).

To create a vaccine candidate for plasmid DNA, a number of conditions need to be investigated, 1) manufacture of the plasmid and the lipid, taking care to remove any impurities, and being able to store them, 2) optimising the expression of the plasmid promoter region, 3) optimisation of the ratios for the cell type that is being targeted, 4) transgene expression occurs, and manages to accumulate within the dendritic cells, 5) stability of transfection, the transgene being present for a length of time, 6) some adjuvant activity, for example CpG present on the plasmid, or adjuvanticity of the cytofectins. Although we have not touched upon parts 5 and 6, we have shown the importance of choosing the right plasmid, of optimising the ratio, and the effect of inappropriate storage of these materials. We have also shown, that in intranasal immunisation, the transgene product can be found within DCs 48 hours post inoculation.

Chapter 4 – Optimising DNA delivery via a novel peptide delivery system

4.1 Introduction

Depending upon the route of immunisation with plasmid DNA, the level of transgene expression has been observed to alter radically. For example, if the route is intranasal (Gao *et al.* 1997; Okada *et al.* 1997), intravenous (Shichiri *et al.* 2003) or intraperitoneal (Lee *et al.* 2002), the naked DNA is rapidly degraded before it can transfect the tissues. In rat sera, plasmid DNA was shown to be degraded within 5 minutes (Yu *et al.* 2001), thus some method to protect DNA from endonuclease degradation and ability to facilitate uptake into cells is required. Chapter 3 discussed the role of cytofectins in plasmid DNA complexes and their ability to increase transfection. However, there were two main disadvantages to this method of transfection, 1) the low level of transfection in non-dividing cells, and 2) potential toxicity of the lipid to the cell. Novel methods of transfection have been investigated to overcome these problems and one of these methods is the use of cell penetrating peptides.

Peptides have been developed to condense plasmid DNA and to target receptors, with the aim of delivering plasmid DNA to the cytosol via an endosomal pathway. The integrin binding domain of the american pit viper snake venom fused to a 16 poly-l-lysine chain has been reported to condense and package the plasmid DNA (Collins *et al.* 2000). The binding of this peptide to the integrin receptor, induces internalisation of the receptor, and targeting to the endosome. However, the limitation for this delivery peptide system is that some mechanism to escape the endosome is required, for optimal transfection to occur.

Cell penetrating peptides have recently been discovered based on the observations that the proteins, HIV tat (Han *et al.* 2001), Antennapedia homeoprotein from Drosophila (Derossi *et al.* 1996) and VP22 of Herpes Simplex Virus type 1 (Elliott and O'Hare 2000) could translocate across the cell membrane into the cytosol of cells. These transduction properties were further defined to a small peptide element within the proteins, that contain basic amino acids (arginine and lysine), which are hypothesised to be important for lipid interactions and/or penetration of the membrane (Schwarze and Dowdy 2000). Peptides synthesised to contain these sequences, have also been reported to demonstrate translocational properties *in vitro* (Derossi *et al.* 1994; Derossi *et al.* 1996). For example, a 16 amino acid sequence, RQIKIWFQNRRMKWKK, comprising of the third alpha helix of the homedomain of Antennapedia, has been shown to be internalised in several cell lines (Mi *et al.* 2000). The properties of these peptides have been examined by fusing the translocation sequence to the sequence of a reporter peptide or protein. One study used β-galactosidase as a reporter, and examined the distribution of the Tat-fused reporter protein following intraperitoneal injection (Fawell *et al.* 1994). This study demonstrated high levels of β-galactosidase activity, in heart, liver and spleen, with low to moderate activity observed in lungs and skeletal muscle.

The exact mechanism of translocation is unknown, current hypotheses suggest that the high positive charge of these peptides, facilitates binding to the negative charge of the lipid bilayer of the cell (Drin *et al.* 2001). An initial binding of these complexes to heparin sulphate has been suggested, similar to the uptake of cationic lipids (Suzuki *et al.* 2002). Following initial binding, translocation of the peptide across the cell membrane is

not clearly understood. It has been hypothesised that these peptides forms a reverse micelle that transverses through the membrane and into the cytosol (Berlose *et al.* 1996). This translocational property has been mapped to the tryptophan at position 6, and a substitution of that amino acid, can abrogate the translocational properties of the peptide (Berlose *et al.* 1996). It has also been reported that transfection with these peptides can occur in an ATP independent manner. At 4°C transfection can occur, suggestive of traversing the cell membrane in a metabolic independent manner. However, other studies comparing uptake at 4°C and 37°C, suggest that both an endocytic as well as a translocation uptake mechanism may operate (Dom *et al.* 2003). The amphipathic conformation of the Antennapedia peptide is considered a critical component of its translocation activity. A simple mutation of one of the amino acids that change this composition, abrogates much of its ability to translocate across the membrane (Dom *et al.* 2003).

There have been recent reports suggesting that the uptake of these peptides is artifactual, and that the highly basic nature recruits the peptides to the nucleus upon vigorous washing or even very mild fixation procedures of the samples (Richard *et al.* 2003). Thus, when examining fluorescently labelled peptide, or fusion proteins, the fixation process ruptures the membranes. This allows the peptides to gain entry, and traffic to the nucleus, due to the electrostatic attraction of the highly negatively charged DNA within the nucleus. Other published reports refute these observations. Firstly, the Antennapedia peptide has been reported to be present in the cytosol during live cell confocal microscopy. The peptide has also been documented to increase transfection efficiency of

adenoviral particles (Gratton *et al.* 2003). Also conjugation of the tat translocation peptide with plasmid DNA has been reported to enhance cellular transfection *in vitro* and *in vivo* (Torchilin *et al.* 2003).

Work reported in the present chapter investigated the efficiency of Antennapedia complexed DNA into transfected cells *in vitro* and compared them to the cationic lipid, GAP-DMORIE:DpyPE, and the Polymolossin peptide. We show that the Antennapedia peptide does transfect cell lines; however, the transfection is very variable, depending on batch to batch variation, as well, as requiring a number of conditions to efficiently transfecting the cells.

4.2 Results

4.2.1 Investigation of the comparative efficiency of cytofectin and peptide mediated DNA transfection *in vitro*.

Plasmid DNA alone is relatively inefficient at cell transfection. Numerous methods have been investigated including use of cytofectins and synthetic peptides. In this study, a peptide derived from the Antennapedia protein was investigated to determine the efficiency of cellular transfection relative to other transfection methods using the plasmid gWiz encoding SEAP as a reporter protein. In all cases, the amount of DNA was kept constant at 1μg and the amount of peptide/cytofectin varied. The effect of the cytofectins, GAP-DMORIE:DpyPE (complexed at 0.5:1, 0.25:1 and 0.13:1 cytofectin:nucleotide molar ratio) and Lipofectamine (1:05, 1:1, and 1:2 (w/w)), were compared to the peptides polylysine-molossin, and Antennapedia. Transfection ratios were calculated based on the optimal ratios derived from the literature or earlier work on the cationic lipids reported in Chapter 3. Since poly-molossin is an integrin binding protein, the complexed DNA requires some method to escape the endosome. To facilitate endosomal escape, chloroquin was incorporated into the transfection formulation at 25μM, 50μM, or 100μM with DNA:peptide (w/w) at 1:3. For comparison lipofectamine was incorporated as an endosomal escape agent at a plasmid DNA:peptide:lipofectamine formulation of 1:3:05, 1:3:1, 1:3:2 (w/w/w). The Antennapedia peptide was tested at the nucleotide:peptide ratios 0.1:1, 0.05:1 and 0.03:1, to initially examine its transfection efficiency. Although cationic lipids demonstrate greater transfection efficiency, the Antennapedia peptide also

transfect the HEK293T cells, to the same efficiency as the polylysine-molossin peptide with chloroquin (Figure 4.2.1).

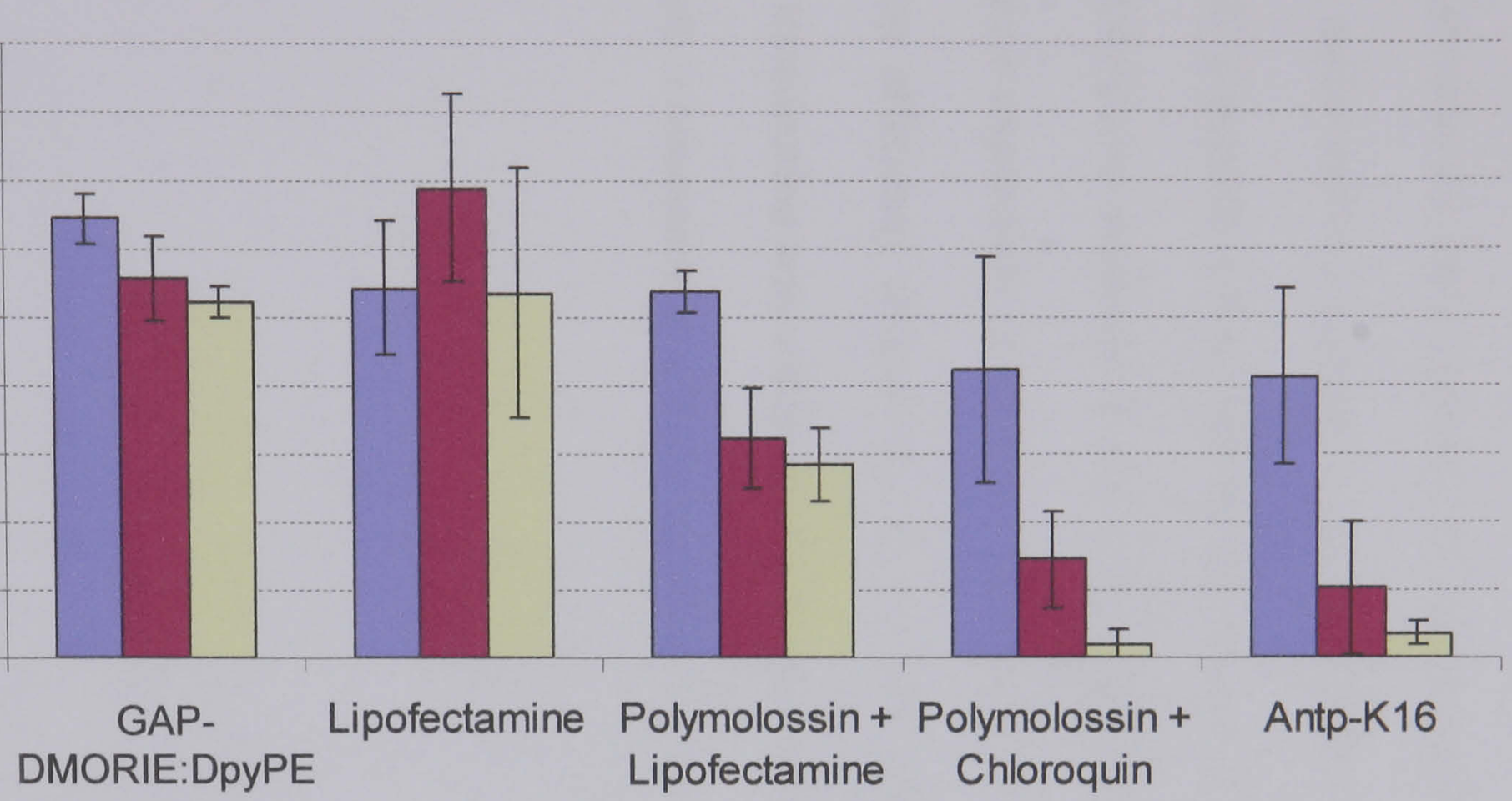


***Figure 4.2.1,* The transfection efficiency of a SEAP encoded plasmid DNA complexed with different delivery agents.** The cationic lipids GAP-DMORIE:DpyPE (molar cytofectin:nucleotide ratios of 0.5:1, 0.25:1 and 0.13:1) and Lipofectamine (1:0.5, 1:1, 1:2 (w/w) DNA:cytofectin), and the peptides polymolosin (with lipofectamine (1:3:0.5, 1:3:1, 1:3:2 (w/w) DNA:peptide: cytofectin) or with chloroquin(1:3 DNA:peptide (w/w) with 25μM, 50μM or 100μM chloroquin) or the Antennapedia peptide(0.1:1, 0.05:1, 0.03:1 molar peptide:nucleotide ratios). Supernatants from HEK293T cells transfected with the SEAP encoded plasmid where examined for the presence of SEAP. The results represent the mean from 5 replicate samples, with the error bars as the standard deviation.

4.2.2 Optimising the transfection efficiencies of peptide based delivery systems.

To maximise the efficiency of transfection with a peptide based delivery system, the peptides Antennapedia (Antp), polylysine-molossin and polylysine-antennapedia (Antp-K16) were each examined at a range of molar:molar ratios of DNA:peptide, to determine the optimal ratio for transfection. The ratios examined were 2.5:1, 1:1, 0.4:1, 0.2:1 and 0.1:1 at a molar ratio of peptide:nucleotide (DNA). The maximal transfection efficiency, for Antp-K16 peptide it was between the ratios 0.5:1 and 0.2:1, for the Antp it was at 1:1, and for polylysine-molossin it was 2.5:1. Each of the peptides induced an increase in reporter gene expression of between 7.1 and 7.5 fold against DNA alone at the maximal transfection efficiency (Figure 4.2.2). For the Antp-K16 peptide, it was observed that maximal transfection was achieved at a higher ratio of nucleotide:peptide, indicating that less peptide is necessary to achieve transfection than with Antp peptide.

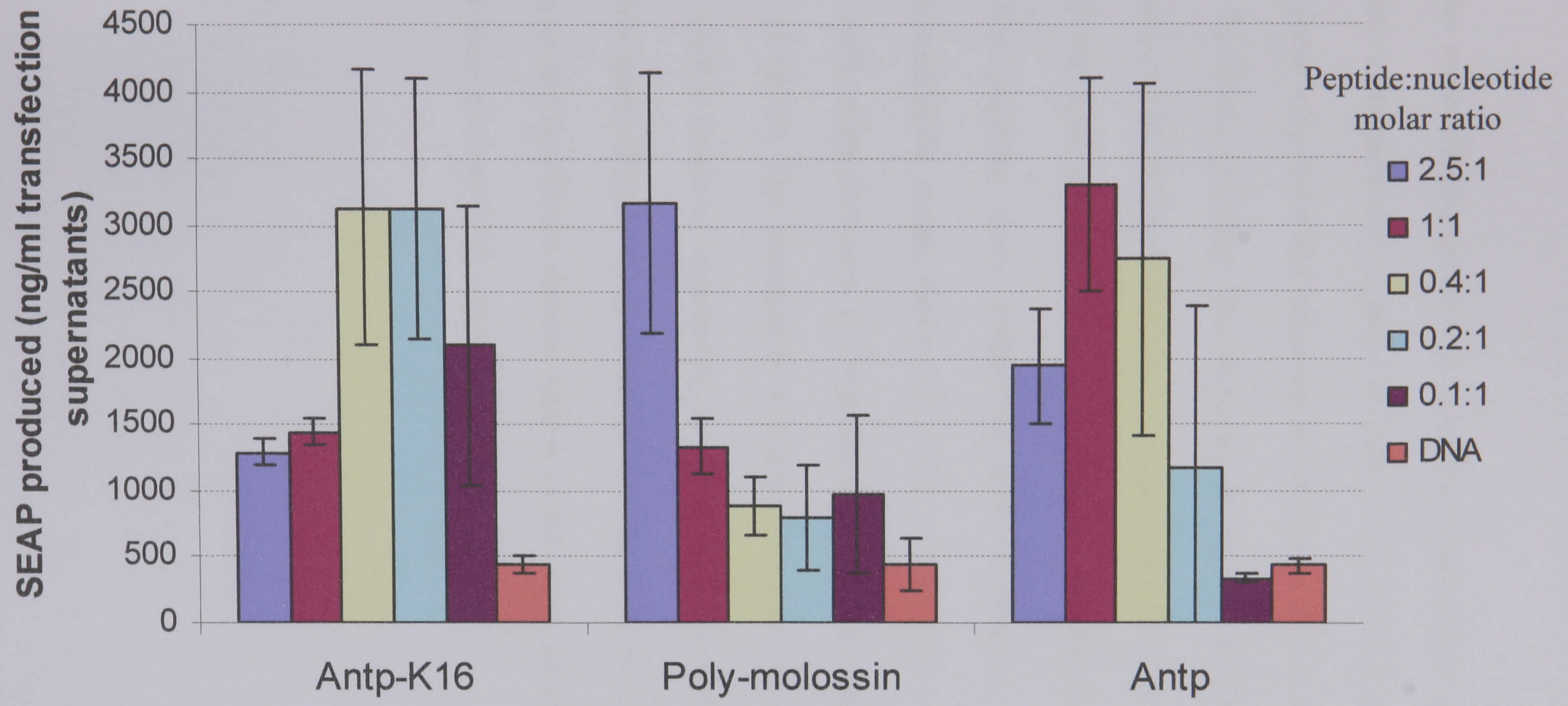


***Figure 4.2.2,* Transfection efficiencies of a SEAP encoded plasmid with peptide delivery agents.** SEAP protein evaluated within the supernatants of HEK293T cells 48 hours post transfection with a SEAP encoded plasmid when complexed with either Antp-K16, Poly-molossin or Antp peptides at the ratios of 2.5:1, 1:1, 0.4:1, 0.2:1 and 0.1:1 peptide:nucleotide molar ratios. The data represents the mean and standard deviation from 5 replicate transfections.

4.2.3 Investigating the effect of freeze thawing on the transfection efficiency of Antp-K16.

One of the most critical parameters to achieve transfection efficiency is the structural composition of transduction peptides (Fischer *et al.* 2000). If the structure is disrupted or changed, these may lower the transfection efficiency. One mechanism by which this can occur is by freeze thawing. We therefore tested the Antp-K16 peptide, after it had undergone a cycle of freeze thawing, for its ability to transfect HEK293T cells. The Antp-K16 was split into two aliquots, one aliquot underwent a freeze thawing cycle at -20°C. The peptides were complexed with a plasmid encoding SEAP at the cytofectin:nucleotide molar ratios 2.5:1, 1:1, 0.4:1, 0.2:1 and 0.1:1 and applied to HEK293 cells. After 48 hours, the supernatants were harvested and investigated for the presence of SEAP. The transfection efficiency is much reduced upon freeze thawing, although similar levels of transfection occur at the 2.5:1 ratio, with a 2.8 fold increase above DNA alone. (Figure 4.2.3) Since, the amount of peptide is reduced, the transfection efficiency of the non-freeze thawed Antp-K16 increases, as opposed to the efficiency of the freeze-thawed Antp-K16, which decreases.

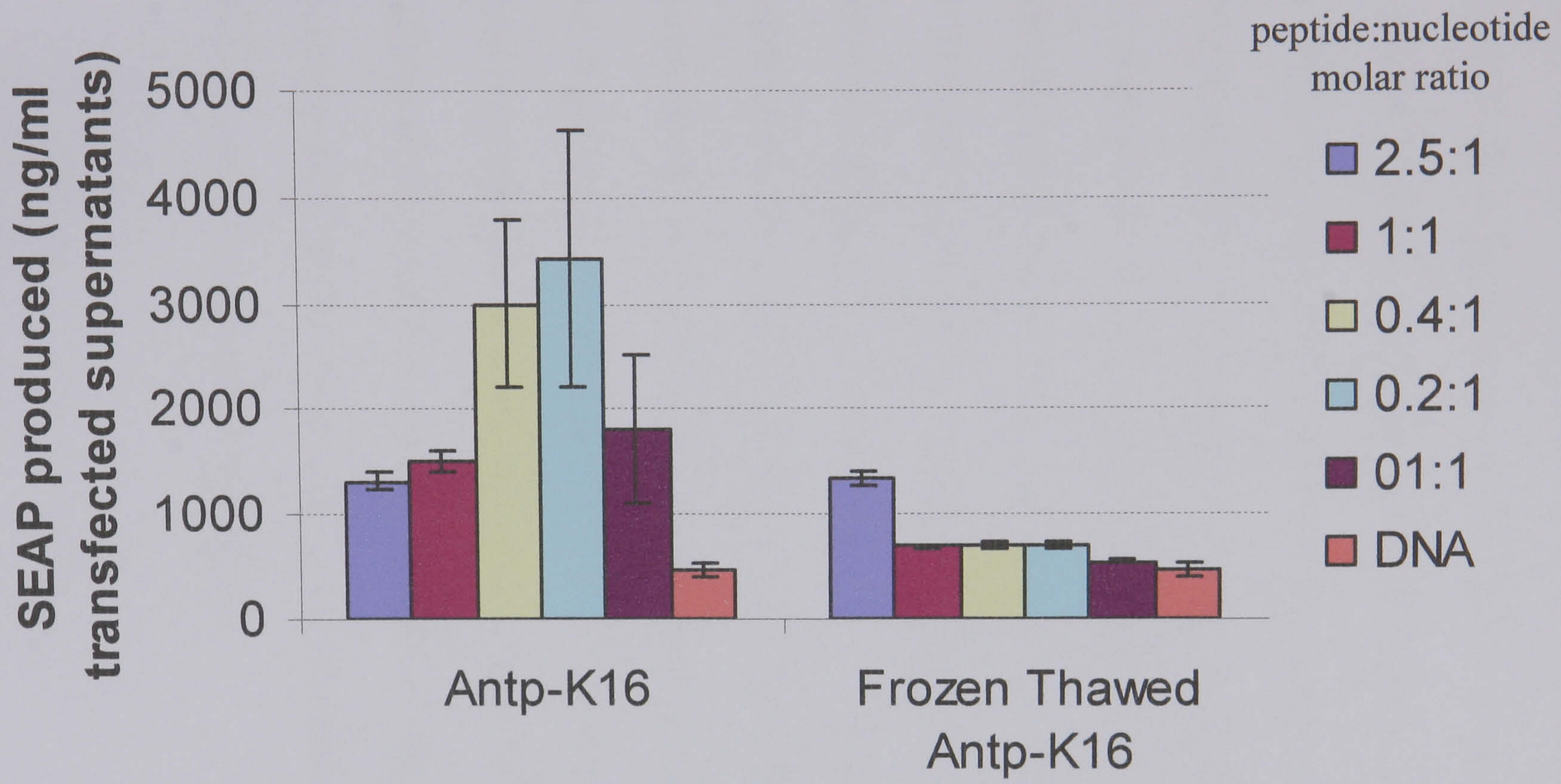


Figure 4.2.3, **Transfection efficiency after Antennapedia has undergone a freeze-thaw cycle.** The concentration of SEAP is reduced in the supernatants of HEK293T cells 48 hours following transfection with a SEAP encoded plasmid when complexed with Antp-K16 that has undergone a cycle of freeze thawing at the molar ratios of 2.5:1, 1:1, 0.4:1, 0.2:1 and 0.1:1 peptide:nucleotide. The results represent the mean and standard deviation from 5 replicate transfections.

4.2.4 Comparison between Antp-K16 and Antp peptides.

Poly-L-lysine has been shown to condense DNA, and protect it from degradation. An Antennapedia peptide was synthesised with 16 lysine residues fused to the carboxy terminus, to act as a carrier of the DNA (Antp-K16). However, since the Antennapedia peptide is highly charged, it is possible that it may act as a carrier without the added poly-L-lysine. Similarly, it is possible that the poly-L-lysine could in some way destabilise the DNA-peptide complex. To investigate these issues, HEK293T cells were transfected with either the Antp-K16 or the Antp peptide, at the ratios of 2.5:1, 1:1, 0.4:1, 0.2:1 and 0.1:1 cytofectin:nucleotide (molar ratios) using the gWiz-SEAP plasmid. Peak expression of secreted SEAP for the Antp-K16 complexed plasmid was observed between 0.2:1 and 0.4:1 ratios, however a greater concentration of Antp peptide was required for the peak SEAP expression which was observed at the 1:1 molar ratio (Figure 4.2.4). By including chloroquin in the transfection, we observed that the amount of SEAP protein produced by the Antp complexed DNA in the presence of chloroquin peaked at the same concentration as the Antp-K16 complexed plasmid DNA, at a ratio of 0.25:1 (Figure 4.2.5). The same concentrations of the Antp-K16 peptide complexed with plasmid DNA was observed to induce peak levels of SEAP production in the presence or absence of chloroquin at the ratios of 0.2:1 to 0.4:1 (Figure 4.2.4 and 4.2.5).

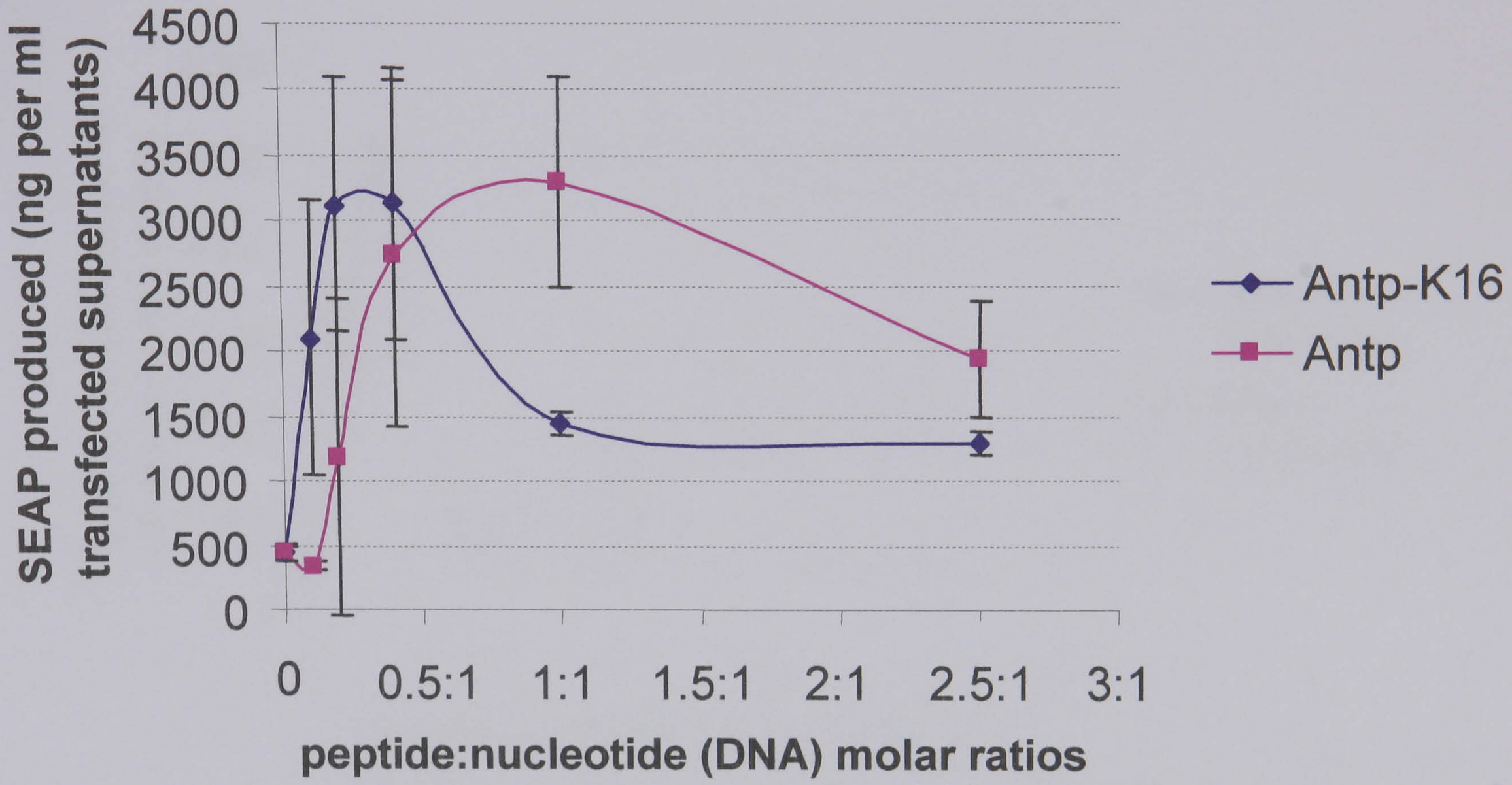


Figure 4.2.4, **Transfection efficiencies of a SEAP encoded plasmid complex with an Antennapedia peptide in the presence or absence of poly-L-lysine.** The presence of SEAP within the supernatants of HEK293T cells 48 hours post transfection with a SEAP encoded plasmid when complexed with either Antp-K6 or Antp peptide at the peptide:nucleotide (DNA) molar ratios of 2.5:1, 1:1, 0.4:1, 0.2:1 and 0.1:1. The data is representative of the means and standard deviations from 5 replicate transfections.

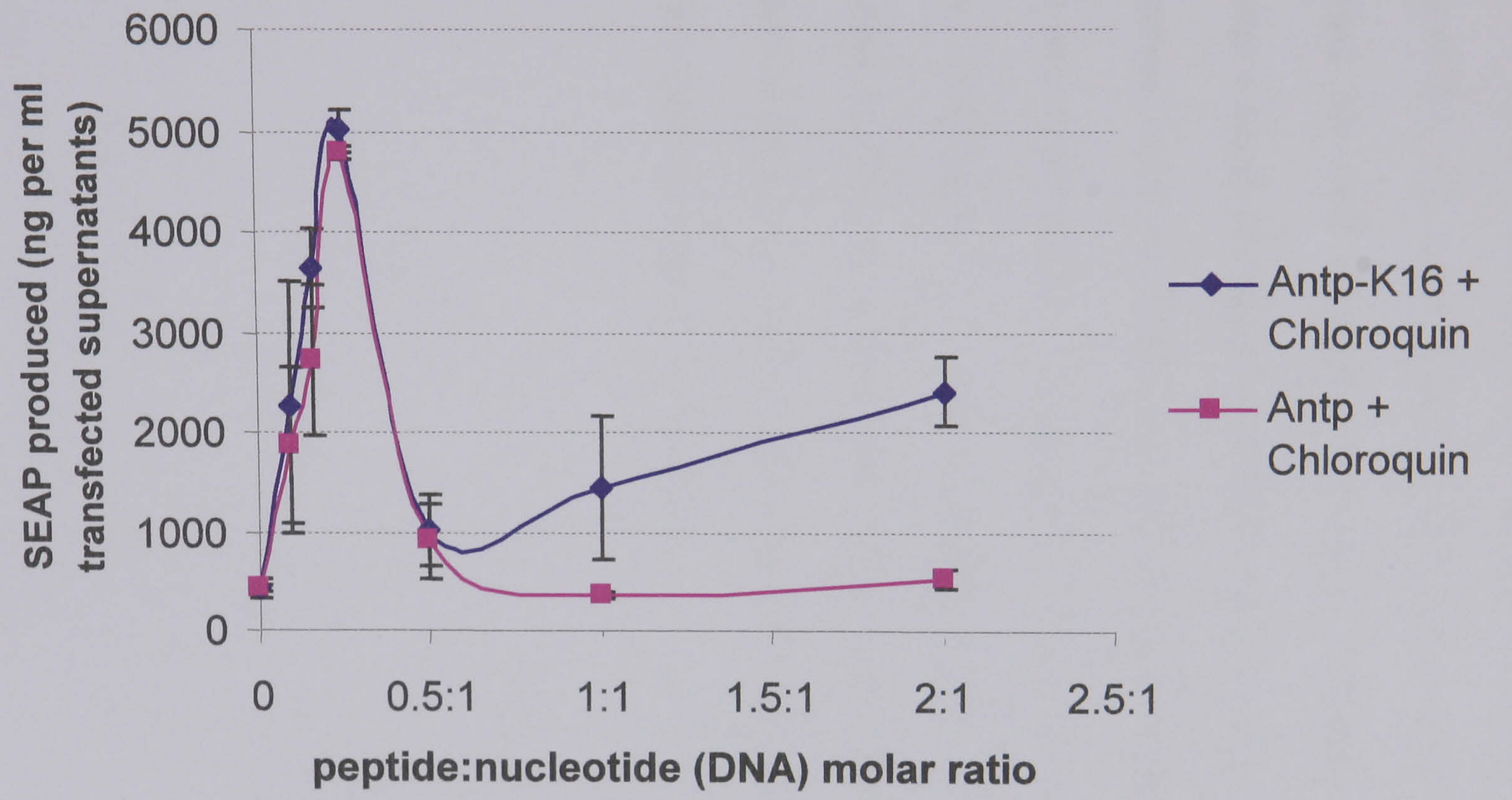


***Figure 4.2.5,* Transfection efficiencies of a SEAP encoded plasmid with Antennapedia peptide in the presence of chloroquin.** Expression of SEAP within the supernatants of HEK293T cells 48 hours post transfection with a SEAP encoded plasmid when complexed with Antp-K16 and Antp peptide at the peptide:nucleotide (DNA) molar ratios of 2.:1, 1.:1, 0.5:1, 0.25:1, 0.17:1, 0.13:1 and 0.1:1 in the presence of 25μM Chloroquin. The data represents the mean and standard deviation of 5 replicate transfections.

4.2.5 Transfection with Antennapedia peptide and a GFP expressing plasmid.

A second plasmid encoded reporter, GFP was used to investigate enhancement of reporter protein expression by the Antp peptide delivery system. HEK293 cells were transfected with a GFP expressing plasmid, gWiz-GFP, at the peptide:nucleotide (DNA) molar ratios of 2:1, 1:1, 0.5:1, 0.25:1, 0.17:1, 0.13:1 and 0.1:1. After 48 hours, the cells were analysed for GFP expression by flow cytometry. The levels of transfection observed, over a range of peptide:nucleotide ratios (Figure 4.2.6) demonstrated the same pattern observed with the SEAP encoded plasmid (Figure 4.2.4 and 4.2.5). Optimal transfection was observed at the ratios of 0.17:1 and 0.25:1, with over 80% of all cells in the culture expressing the GFP transgene. Over 25% of cells at a 0.17:1 peptide:nucleotide ratio expressed GFP with a mean fluorescent intensity greater than 600 indicative of high level of GFP expression, which may relate to transfection of each cell with more than one copy of the plasmid.

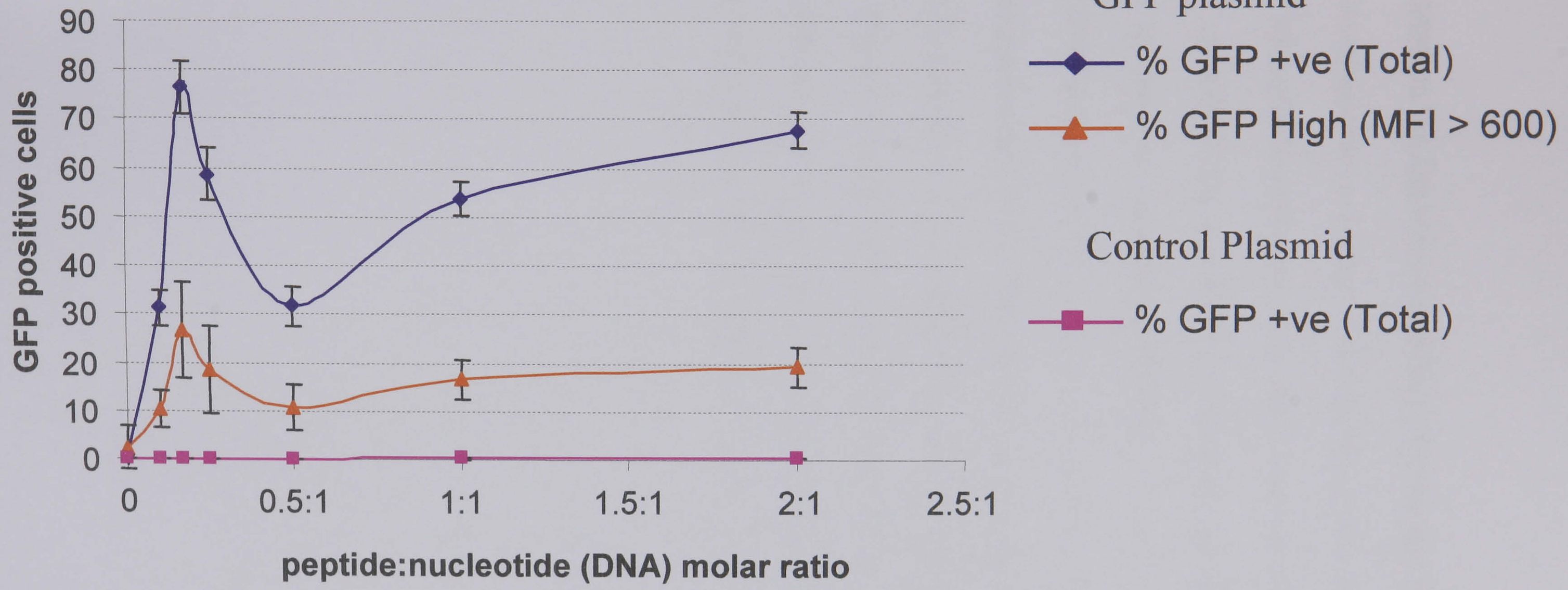


Figure 4.2.6, **Transfection efficiency of a GFP encoded plasmid when complexed with an Antennapedia peptide.** GFP expression within HEK293T cells 48 hours post transfection with a GFP encoded plasmid complexed with Antp-K16 at the peptide:nucleotide (DNA) molar ratios of 2:1, 1:1, 0.25:1, 0.17:1, 0.13:1. 0.1:1. GFP expression within total cells (◆), and cells with an MFI greater than 600 (▲) compared to an irrelevant plasmid (■). Data represents the means and standard deviation from 5 replicate transfection.

4.2.6 Investigating the Transfection efficiencies of a new batch of Antp-K16 peptide.

To further investigate the properties of this peptide, a new batch had to be synthesised by Cambridge Research Biochemicals, the same company who initially synthesised the peptide. To examine if the transfection efficiencies generated by the new batch was comparable to previous transfection, HEK293 T cells were transfected with Antp complexed DNA at the ratios 4:1, 2:1, 1:1, 0.5:1, 0.25:1, 0.17:1, 0.13:1, 0.1:1, 0.8:1, and 0.7:1 peptide:nucleotide (DNA). The optimal transfection was observed at the 0.5:1 ratio (Figure 4.2.7), compared to the optimal ratio previously observed to be between 0.17:1 and 0.25:1 (Figure 4.2.4 and 4.2.6). However, the production of the SEAP reporter was also much reduced, with the maximal transfection being observed to be 32 fold less than the optimal transfection previously reported in Figure 4.2.4.

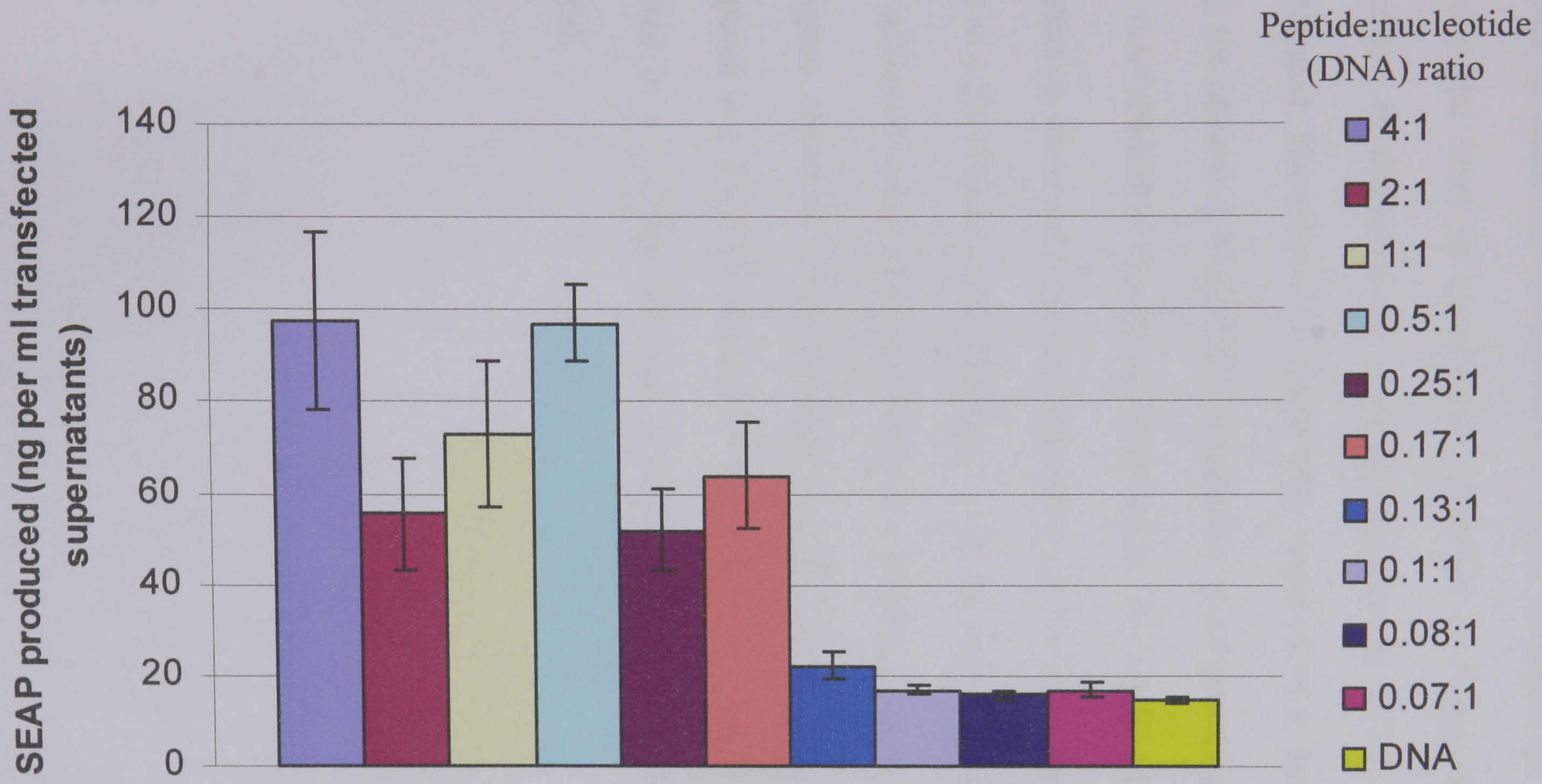


Figure 4.2.7, Transfection of SEAP encoded plasmid DNA complexed with Antennapedia. A newly synthesised Antennapedia peptide was examined for its transfection efficiency by complexing with DNA at the peptide:nucleotide molar ratios of 4:1, 2:1, 1:1, 0.5:1, 0.25:1, 0.17:1, 0.13:1, 0.1:1, 0.08:1, and 0.07:1. After 48 hours, supernatants from HEK293T cells encoded with the DNA peptide complex was examined for its SEAP activity. The data represents the mean and standard deviations from 5 replicate transfections.

4.2.7 Changing the transfection formulation can affect the efficiency of transfection.

The conditions for complexing the peptide are crucial, for example the correct pH is required to allow for conformation and to maintain the correct charge. To try and improve the level of transfection observed in Figure 4.2.7, different buffers were investigate to formulate the DNA and peptide (at 0.17:1 peptide:nucleotide ratio) was investigated. The effects of 10mM Tris, 1mM HEPES, Saline and PBS were compared using the plasmid gWiz-SEAP, to evaluate SEAP protein expression following delivery with GAP-DMORIE:DpyPE or DNA alone. The results show that the absolute level of transfection observed is reduced irrespective of delivery formulation compared to figures 4.2.1 to 4.2.6 (Figure 4.2.8). However, it can be clearly observed that optimal transfection was achieved using Tris to buffer the complexing of Antp with DNA. The level of transgene observed was increased 5.4 fold above that observed with DNA alone, compared to a 3.6 fold increase observed using saline as buffer. Inclusion of PBS, only resulted in a 1.4 fold increase in reporter protein production above the DNA alone control.

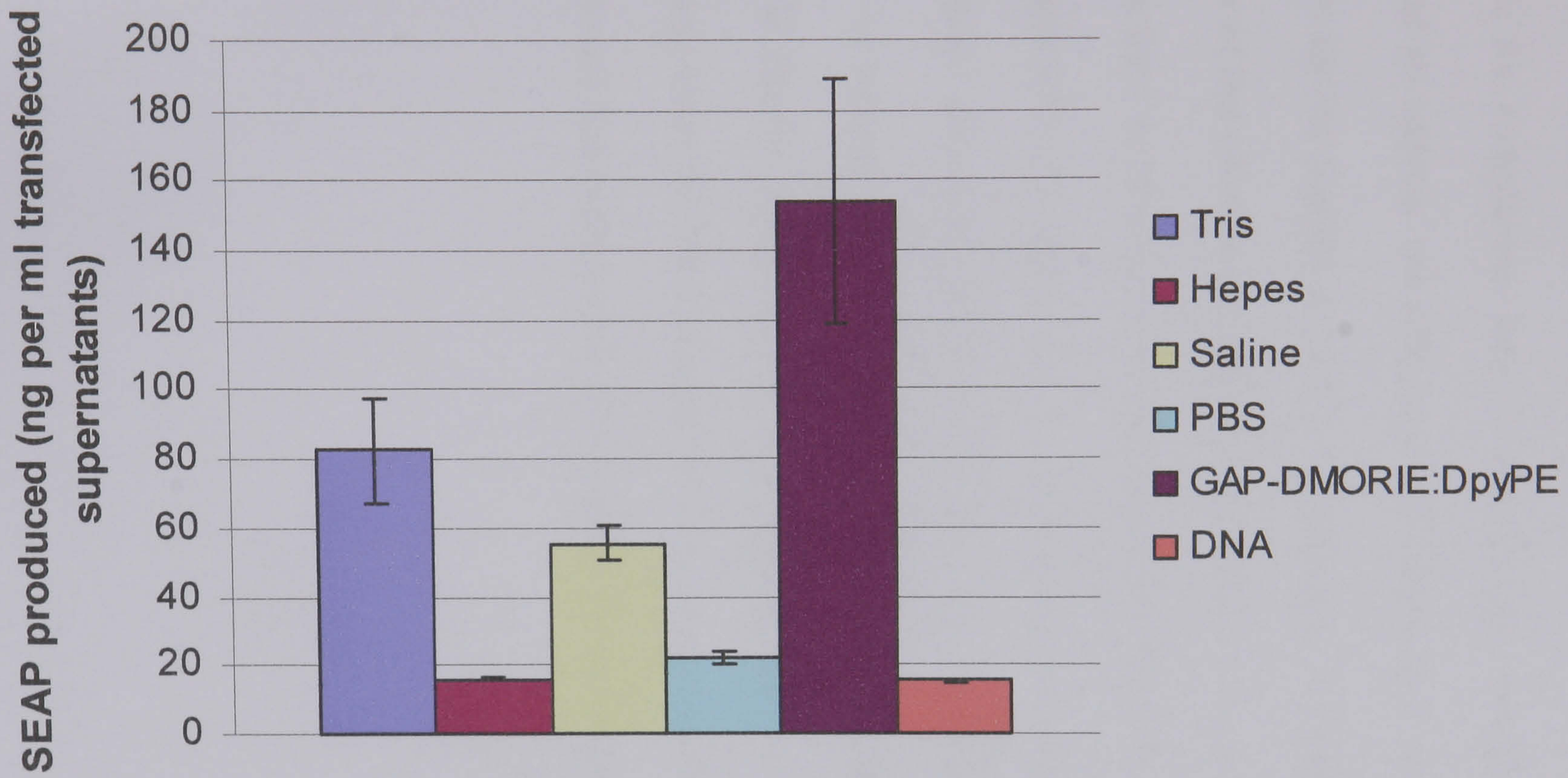


Figure 4.2.8, Transfection efficiency of a SEAP encoded plasmid when complexed with a new batch of Antennapedia in a range of buffers. The Antp-K16 peptide was complexed at the molar ratio of 0.17:1 peptide:nucleotide in the presence of 10mM Tris, 2mM Hepes, Saline or PBS. GAP-DMORIE:DpyPE complexed plasmid DNA was used as a positive control at the molar:molar ratio of 6:1. Supernatants from HEK293T cells transfected with each tested for the presence of SEAP. Supernatants from plasmid complexed GAP-DMORIE:DpyPE transfections were diluted 1 in 10 with media. Results represent the mean and standard deviation of 5 replicate transfections.

4.2.8 Time course for optimal complexing of the Antennapedia-DNA complex.

There have been reports in the literature that the Antennapedia peptide can bind very efficiently to plastic. In such a scenario, then the amount of Antennapedia peptide that is available for complexing may be significantly reduced. Therefore a time course was examined to address the efficiency of transfection, by changing the duration of time available for the peptide and DNA to complex. The time course ranged from immediate addition of the DNA and peptide to the target cells, or incubating the peptide and DNA complex for 5 to 30 minutes before addition to the cells, at the peptide:nucleotide (DNA) molar ratio of 0.17:1 (figure 4.2.9). These complexing conditions were compared to DNA transfection with GAP-DMORIE:DpyPE, and DNA alone as controls. Although the amount of reporter protein is reduced with the new peptide batch compared to the GAP-DMORIE:DpyPE control, there is a noticeable loss 7.1 fold decrease in transfection efficiency, when the DNA and peptide are complexed for 5 mins prior to addition to the indicator cell line compared to direct addition of the complex.

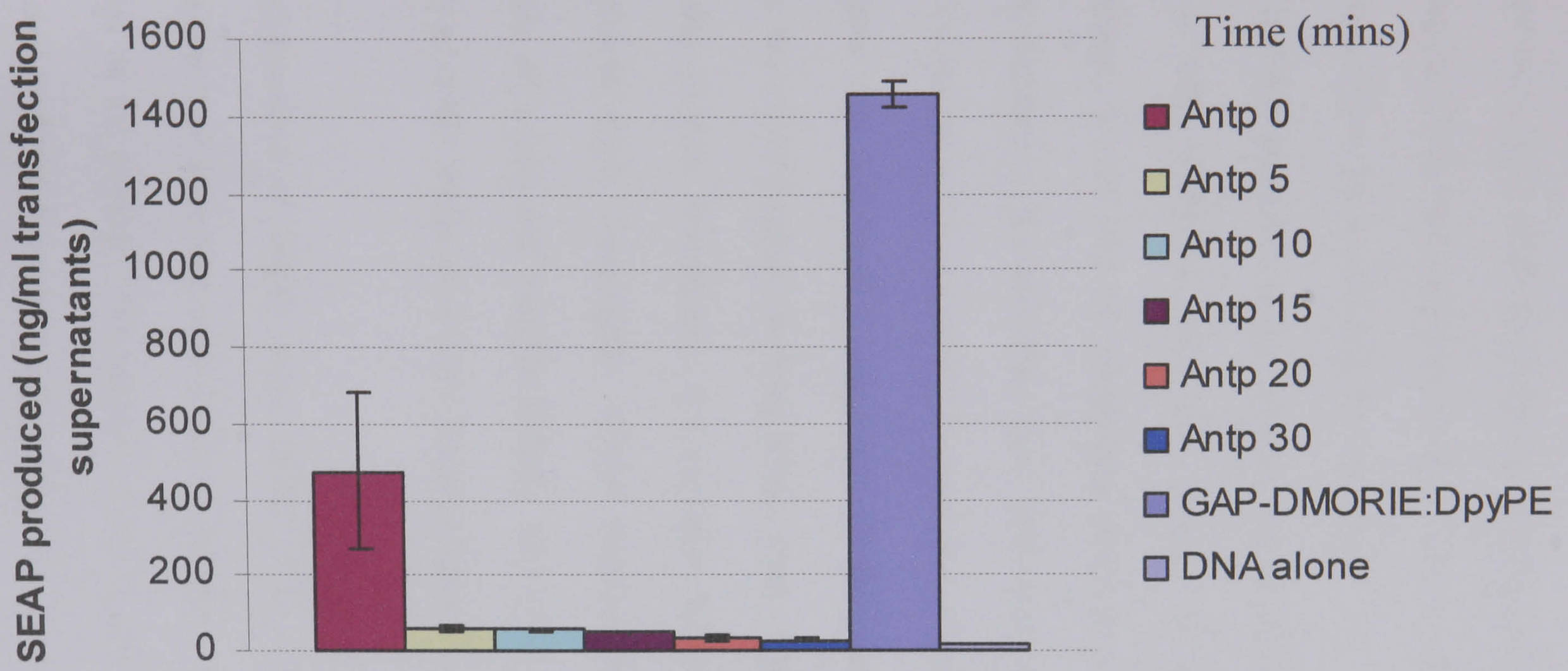


Figure 4.2.9, **The effect of DNA-peptide complexing time on the efficiency of Antennapedia mediated DNA transfection in HEK293 cells.** Antennapedia peptide was complexed at the molar ratio of 0.17:1 (peptide:nucleotide) and was added to HEK293T cells after being complexed for 5, 10, 15, 20 or 30 minutes, or as soon as the peptide was added to the DNA. GAP-DMORIE:DpyPE at the molar:molar ratio of 0.17:1 (cytofectin:nucleotide) was transfected into HEK293 cells as a positive control, and DNA alone as a negative control. The data represents the mean and standard deviations from 5 replicate transfections.

4.3 Discussion

There are two major problems inherent in plasmid DNA immunisation. These are: 1) protecting the DNA from degradation by serum or tissue endonucleases; and 2) efficiency of plasmid DNA internalisation by the target cell. A well documented procedure to overcome these problems is to complex the plasmid DNA with cytofectins. However, there are also problems with this method of transfection. There are potential disadvantages to the use of cytofectins since they have been observed to primarily transfect dividing cells, and they have also been attributed with varying degrees of cellular cytotoxicity. Consequently, a novel, non-toxic method of DNA delivery has been investigated that will equally target both dividing and non-dividing cells. This method involves the use of peptides derived from a class of proteins that can directly translocate across the plasma membrane, for example HIV-Tat protein or the Antennapedia homeoprotein from *Drosophila*. These peptides have been reported to cross the membrane of a cell and transfer directly into the cytoplasm, bypassing the endosome (Joliot *et al.* 1991; Joliot *et al.* 1991; Bloch-Gallego *et al.* 1993; Le Roux *et al.* 1993).

The incorporation of highly basic amino acid sequences from certain translocation peptides has been reported, with much of the early work investigating the translocation properties of the peptide. The ability of these peptides to cross the plasma membrane when fused to large macromolecules, gave rise to the idea of complexing the peptides with plasmid DNA. Although within the literature, very little work has examined the transgene product resulting from transfection with plasmid DNA complexed with the

Antp peptide, previous studies have reported an increase in the efficiency of Antp complexed viral particles (Gratton *et al.* 2003), and Antp complexed oligonucleotides (Dom *et al.* 2003). All point to the efficient movement of these large macromolecules into the cytosol, and even into the nucleus. The observed cellular entry of these peptides across the plasma membrane reported in the literature, gives credence to the data we have shown in this thesis, that not only can the translocation peptide enter the cytosol, but can also carry large plasmid DNA molecules, that will transcribe and translate the encoded transgene in the transfected cell. The Antennapedia peptide complexed to a GFP plasmid (figure 4.2.6) was shown to transfect 75% of the cells at the optimal ratio of transfection. Of those 25% demonstrate high levels of gene expression (MFI>400), indicative that the cell has either efficiently produced the transgene, or has been transfected with more than one copy of the plasmid.

Recent studies by other investigators have examined the ability of the Tat peptide to carry plasmid DNA across the plasma membrane and transfect cells (Eguchi *et al.* 2001; Snyder and Dowdy 2001; Ignatovich *et al.* 2003). The results observed are very similar to the results observed within this study. The transduction peptides do increase the level of transgene produced compared to naked DNA, but the rate of transfection is reduced, compared to the transfections observed with the cationic lipids mediated gene delivery.

Two Antennapedia peptides were investigated in this study; one of them only contained the 16 amino acids that induced translocation (Antp). However, the other contained 16 lysine residues (Antp-K16) to encapsulate the plasmid DNA. The presence of the lysine

induced lower the concentrations of Antp-K16 to transfect efficiently resulted in higher level of reporter gene production than the comparative concentration of the Antp peptide. (Figure 4.2.4). This is potential due to the lysine residues either facilitating uptake to the cell, or by protecting the plasmid DNA while it is escaping the endosome. The Antennapedia peptide is thought to bind to the cell membrane by charge related means (Drin *et al.* 2001), therefore it is unsurprising that the Antp-K16 which has a greater net positive charge would bind to the membrane stronger, and thus facilitate a greater uptake of the peptide.

The Antp peptides have been reported to translocate to the cytosol in an endosome independent manner (Berlose *et al.* 1996), therefore upon addition of chloroquin an endosomal disruption agent, any DNA-peptide complex that is in the endosome will be released into the cytosol. Thus DNA entering directly into the endosome (via receptor mediated endocytosis) will be released into the cytosol, resulting in a significant increase in the level of reporter protein produced. In this study, Antp-K16 peptide complexed plasmid DNA transfected cells at a lower concentration than the Antp peptide in the absence of chloroquin (Figure 4.2.4). However, in the presence of chloroquin the concentration of Antp peptide and Antp-K16 required to optimally transfect cells with plasmid DNA was identical (Figure 4.2.5). This suggests that the complex is targeted to the endosome as has been recently reported (Koppelhus *et al.* 2002; Richard *et al.* 2003) and the Antennapedia core peptide allows the plasmid DNA to escape from the endosome by an unknown mechanism. The presence of the poly-l-lysine protected the plasmid DNA

from degradation, thus lower concentrations of the Antp-K16 peptide facilitated transfection in the absence of an endosomal disruption agent.

The work in this chapter has further demonstrated that secondary structure is critical for the transfection efficiency of Antp based peptides and is in agreement with earlier studies (Berlose *et al.* 1996). Freeze thawing of the peptide and suboptimal buffering (pH) of the complexing media was observed to reduce transfection efficiency. Both parameters directly affect the secondary structure of this highly basic protein. Although peptides are expensive to manufacture, the ability to transfect non-dividing cells is a beneficial attribute over lipid based cytofectins currently under investigation. Further studies into the properties of these peptides, especially with recent reports that the initial investigations into the transfection properties were artifactual (Richard *et al.* 2003) is essential.

Chapter 5 – Investigation of CCL20 as a potential Genetic Adjuvant

5. 1 Introduction

Naked plasmid DNA has been shown to be efficacious in eliciting immune response within murine models. However, the quantity of DNA required for efficient primate vaccination is too large to be practical or financially viable. A possible approach is to increase the efficacy of plasmid DNA vaccination is the use of adjuvants. Examples of molecules that display adjuvant like activity include the chemokines, CCL5 (RANTES) (Kim *et al.* 2000; Sin *et al.* 2000; Oh *et al.* 2003), CCL3 (MIP-1α) (Kim *et al.* 1998; Kim *et al.* 2000; Youssef *et al.* 2000). These molecules recruit DCs and monocytes in a concentration dependant manner. Alternatively, it may be possible to increase the total number of DCs in the local area of immunisation by the use of growth factors, for example GM-CSF (Iwasaki *et al.* 1997; Sin *et al.* 1998; Operschall *et al.* 1999). One major advantage of these protein adjuvants is that they can be encoded by the plasmid DNA vaccine itself and therefore co-delivered with antigen.

In 1997, a novel CC chemokines, CCL20, was first identified using BLASTN and TBLAST searches against GENBANK using cDNA and protein sequences of chicken lymphotactin and other chemokines (Rossi *et al.* 1997). CCL20, also known as LARC (Liver and activation-regulated chemokine), Exodus-2 and MIP-3α. The CCL20 gene was mapped to the D2S159 chromosomal marker at chromosome 2q33-q37 by *in situ* hybridisation. Two clones encoding the entire human CCL20 gene were isolated from a yeast artificial chromosome (YAC) library (Hieshima *et al.* 1997). Sequence analysis showed a 20-28% homology between CCL20 and other CC chemokines family members.

CCL20 expression was demonstrated within lymph nodes, appendix, PBL, foetal liver, foetal lung (Hieshima *et al.* 1997; Rossi *et al.* 1997), and also shown to be upregulated at inflamed epithelial surfaces (Power *et al.* 1997) and by infected macrophages (Matikainen *et al.* 2000; Abiko *et al.* 2003)

Analysis of the biological activity of CCL20 showed it was a potent chemoattractant for memory T cells, naive B cells, immature dendritic cells and Langerhan' cells. It was also shown to be weakly chemotactic for granulocytes. (Hieshima *et al.* 1997; Dieu *et al.* 1998; Charbonnier *et al.* 1999; Liao *et al.* 1999; Brandes *et al.* 2000; Dieu-Nosjean *et al.* 2000; Matsui *et al.* 2001; Hosokawa *et al.* 2002). Therefore, it was suggested that CCL20 represented a promising candidate as a genetically encoded adjuvant.

Exposure of the murine monocytoid J774 cell line to lipopolysaccharide (LPS) induced the expression of CCL20. However, TNFα, IL-1β, IFN-γ and IL-4 were unable to induce CCL20 expression in this cell line (Tanaka *et al.* 1999). In one study, CCL20 mRNA was shown to be present within intestinal tissues and upregulated upon treatment with LPS. This correlated with an increased production of CCL20 protein. (Tanaka *et al.* 1999).

The specificity of CCL20 receptor binding was determined using a panel of stably transfected HEK293T cell lines, each of which expressed a single chemokine receptor family member. CCL20 was shown to be unable to bind to either CCR1-5 or CXCR1-2 (Liao *et al.* 1997). This was taken as evidence that CCL20 bound to a unique chemokine receptor. The cognate receptor for CCL20, was identified as CCR6 (initially called

SRL22) (Greaves *et al.* 1997; Liao *et al.* 1997; Power *et al.* 1997). The CCR6 receptor was identified by RT-PCR, using degenerate oligonucleotide primers based on the sequence homology of known chemokine receptors, and was reported to be the specific receptor for CCL20 (Power *et al.* 1997). Out of a panel of 25 chemokines tested CCR6 specifically bound CCL20. CCR6 showed differential expression on human dendritic cells. DCs derived from CD34+ cord blood precursors were $CCR6^{+ve}$ whilst DCs derived from human peripheral blood monocytes were $CCR6^{-ve}$. (Greaves *et al.* 1997). Messenger RNA for murine CCR6 was also shown to present within B cells, CD8- splenic dendritic cells and CD4+ T cells when sorted by flow cytometric purified populations, and analysed by RT-PCR. (Varona *et al.* 1998).

CCL20 is currently a member of a unique group of chemokines that are not promiscuous in their binding and are specific for a single receptor. CCL20 exhibits a similar structure to most chemokines, with a three-stranded beta-sheet and an overlying alpha-helix (Perez-Canadillas *et al.* 2001). The chemokines differ in the conformation of their N-terminal DCCL motif, which has been postulated to account for receptor specificity. As could the narrowness of the groove between the N-loop and the beta2-beta3 hairpin as compared to other chemokines (Perez-Canadillas *et al.* 2001). In support of this hypothesis, human beta defensin 2 (a non-chemokine molecule that can bind to CCR6), demonstrates similar structural features to CCL20 in the absence of any significant amino acid sequence homology (Yang *et al.* 1999; Biragyn *et al.* 2001; Perez-Canadillas *et al.* 2001).

CCL20 is thought to play a role in the homeostasis of DC trafficking (Dieu-Nosjean *et al.* 1999; Sozzani *et al.* 1999; Caux *et al.* 2002). Immature DCs express CCR6, which upon DC maturation, is lost and replaced with CCR7 (Dieu *et al.* 1998). In transwell migration assays, DCs derived from CD34+ hematopoietic progenitor cells (HPCs) or monocytes, expressed CCR6 and migrated to CCL20 in a concentration dependent fashion. If these DC cultures were stimulated with LPS, TNFα, or CD40L they downregulated their CCR6, and upregulated their CCR7 expression, and subsequently failed to migrate towards CCL20 (Dieu *et al.* 1998).

Iwasaki and Kelsall, 2000 defined the DC populations present within the Peyer's patch and provided evidence that CCL20 has a functional role in DC homeostasis *in vivo*. They identified 3 subsets of DCs in the Peyer's patch; myeloid DCs ($CD11c^+$ $CD11b^+$), lymphoid DCs ($CD11c^+$ $CD8\alpha^+$) and a third subset defined as $CD11b^-$ and $CD8\alpha^-$. Histological analysis of Peyer's Patches localised CCL20, CCR6 and the myeloid DCs to the subepithelial dome (SED). In contrast, lymphoid DCs localized to the T cell-rich inter-follicular region (IFR), which in turn correlated with the expression of CCR7 and its cognate chemokine CCL19 (Iwasaki and Kelsall 2000).

This *in vivo* histological evidence supports the hypothesis that CCR6 ligands such as CCL20 may recruit immature DCs to the site of antigen exposure. This may in turn, result in an enhanced level of priming and therefore an enhanced immune response. Plasmid encoded CCL20 may therefore increase the efficacy of plasmid DNA vaccines by local expression at the site of immunisation. Further evidence to support this theory

came from studies of tumour immunotherapy. When injected into subcutaneous tumours, adenovirally expressed CCL20 led to the recruitment of DCs *in vivo* (Fushimi *et al.* 2000).

This study examines the potential of CCL20, expressed from a non-viral vector, as a genetic adjuvant. We show the cloning of CCL20, validation of its biological function, and have examined the capacity of plasmid encoded CCL20 to recruit DCs to the site of inoculation. (NALT) *in vivo*. In order to maximise expression from plasmids, the information derived from chapter 3, "Optimisation of DNA delivery using cytofectins" was utilised to enhance transfection efficiency in all the studies in this chapter.

5.2 Results

5.2.1 Validation of CCR6 and CCL20 expression within NALT.

Our strategy was based upon the expansion of priming through the recruitment of DC populations to the site of immunisation. It is essential that the adjuvants investigated are biologically active within the mucosa. Therefore, we investigated CCL20 and CCR6 expression within NALT. NALT was harvested from mice and a 294 bp band (CCL20) and a 548 bp band (CCR6) mRNA was demonstrated to be present by RT-PCR (Figure 5.2.1) HPRT and no reverse transcription controls showed that these bands were specific for CCL20 and CCR6 cDNA and not due to genomic DNA contamination. CCR6 protein was demonstrated to be present by flow cytometry in NALT using polyclonal rabbit α mouse CCR6, a kind gift from Christine Powers, Serono Pharmaceuticals, Switzerland (Figure 5.2.2).

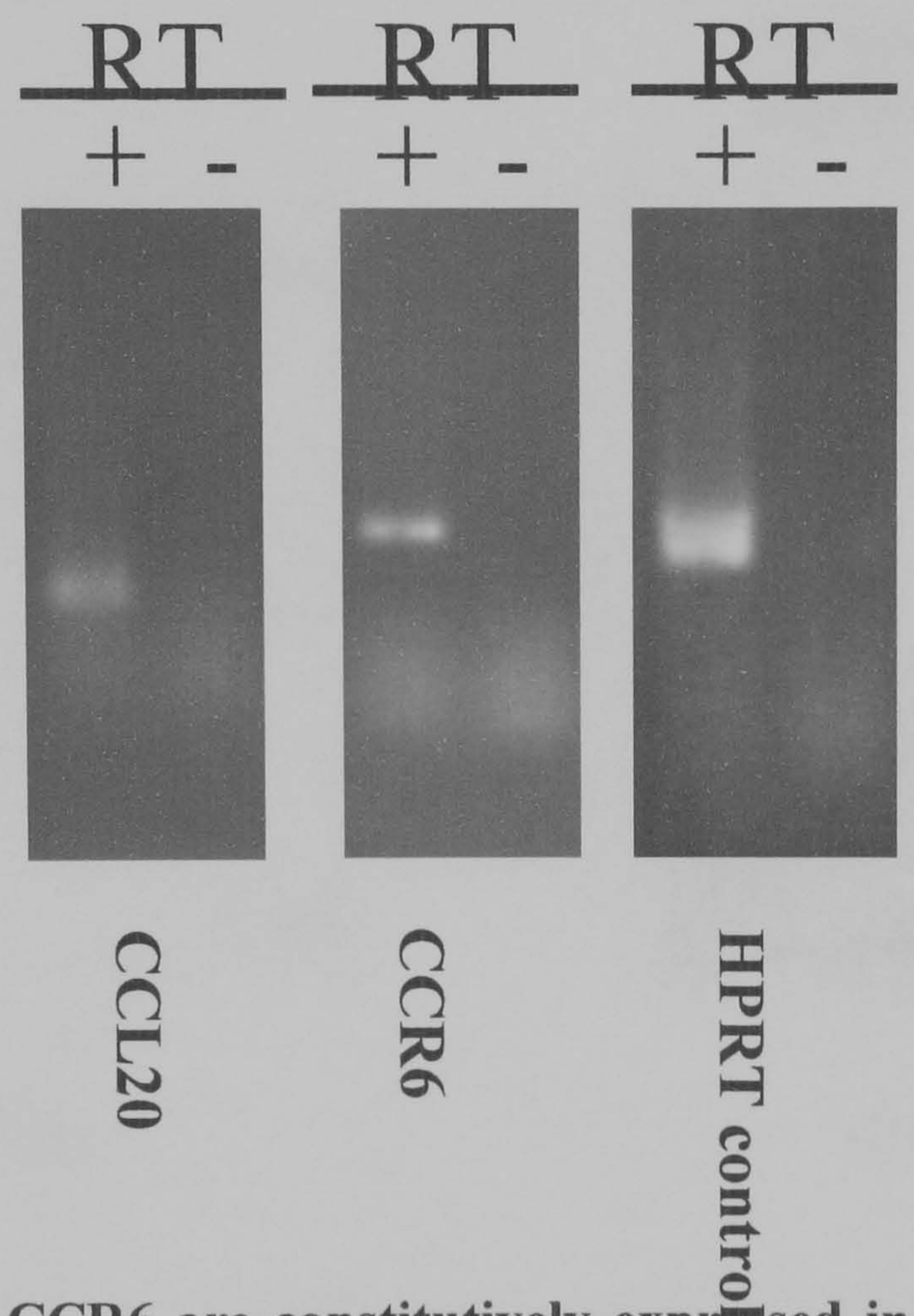


***Figure 5.2.1*, CCL20 and its receptor CCR6 are constitutively expressed in NALT.** Nasal tissue was pooled from six naïve mice, homogenised. mRNA was extracted using TRIZOL and QIAGEN RNAeasy columns. cDNA was synthesised by reverse transcription and primers were used to amplify by PCR, CCL20, CCR6 and hypoxanthine phophoribosyl transferase (HPRT, housekeeping) amplicons.

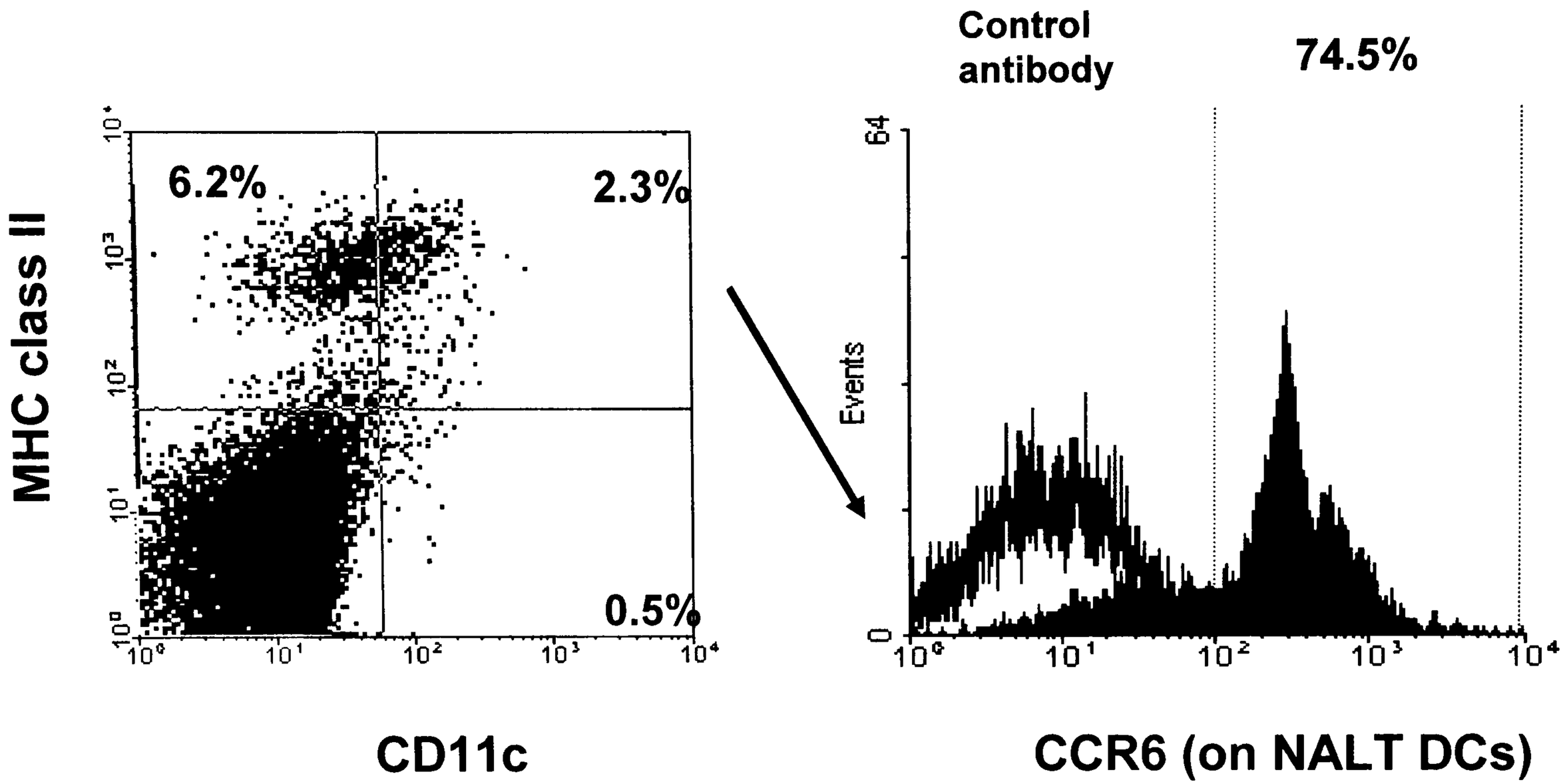


***Figure 5.2.2*, Expression of CCR6 in NALT DC confirmed by flow cytometry.** NALT from 5 naive mice was harvested and a single cell suspension obtained. Cells were stained for MHC class II, CD11c and CCR6. A live gate was defined based on FSC vs SSC, and then examined for CD11c, MHC class II expression. MHC class II^+ $CD11c^+$ cells were gated to show the level of CCR6 expression.

5.2.2 Verification of CCL20 expression

Murine CCL20 was cloned into the expression vector pcDNA3.1^{+} by Dr Patricia Novelli. Murine CCL20 was identified using an EST clone, and inserted into the plasmid pcDNA3.1 between the EcoRV and the BamHI restriction enzyme sites (Figure 5.2.3). Once the sequence was confirmed the expression of the plasmid was examined *in vitro*. pcDNA3.1-CCL20 was complexed with DMRIE:DOPE at a cytofectin:nucleotide molar ratio of 0.17:1 and applied to HEK293 cells. After 48 hours, the supernatants were harvested and separated on a 15% SDS-PAGE and assayed by Western blot for the presence of the protein. The western blot shows the presence of CCL20 using a rat polyclonal antibody (Figure 5.2.4), and using a mouse polyclonal antibody (Figure 5.2.5).

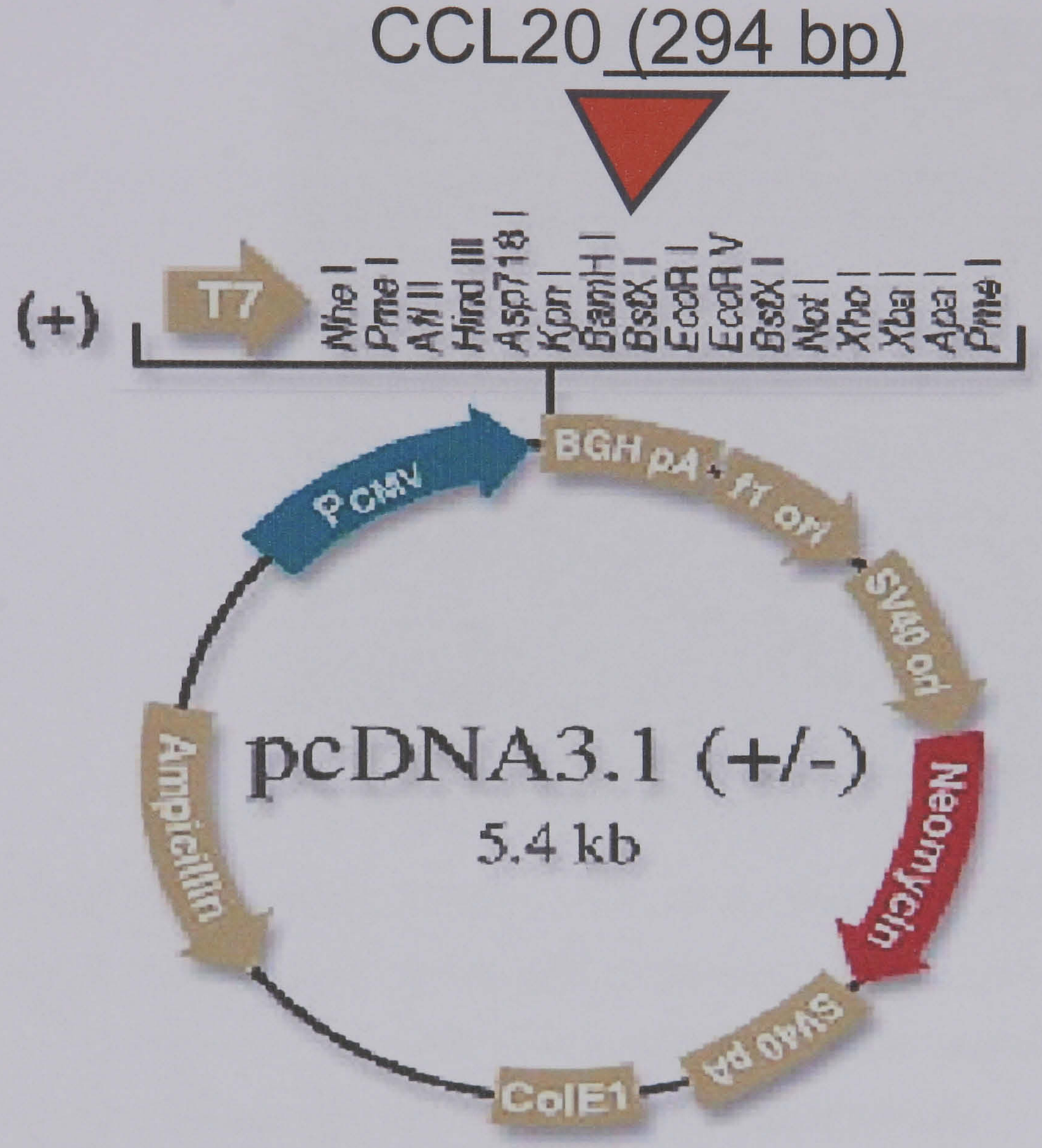


Figure 5.2.3, The sub-cloning of CCL20 into a mammalian expression vector (pcDNA-CCL20). *Primers were designed to amplify CCL20 cDNA excluding the 5' and 3' untranslated regions. The amplified insert was subcloned into the mammalian expression vector pcDNA3.1+. Clones were confirmed positive by restriction digestion, PCR amplification and full-length sequencing.*

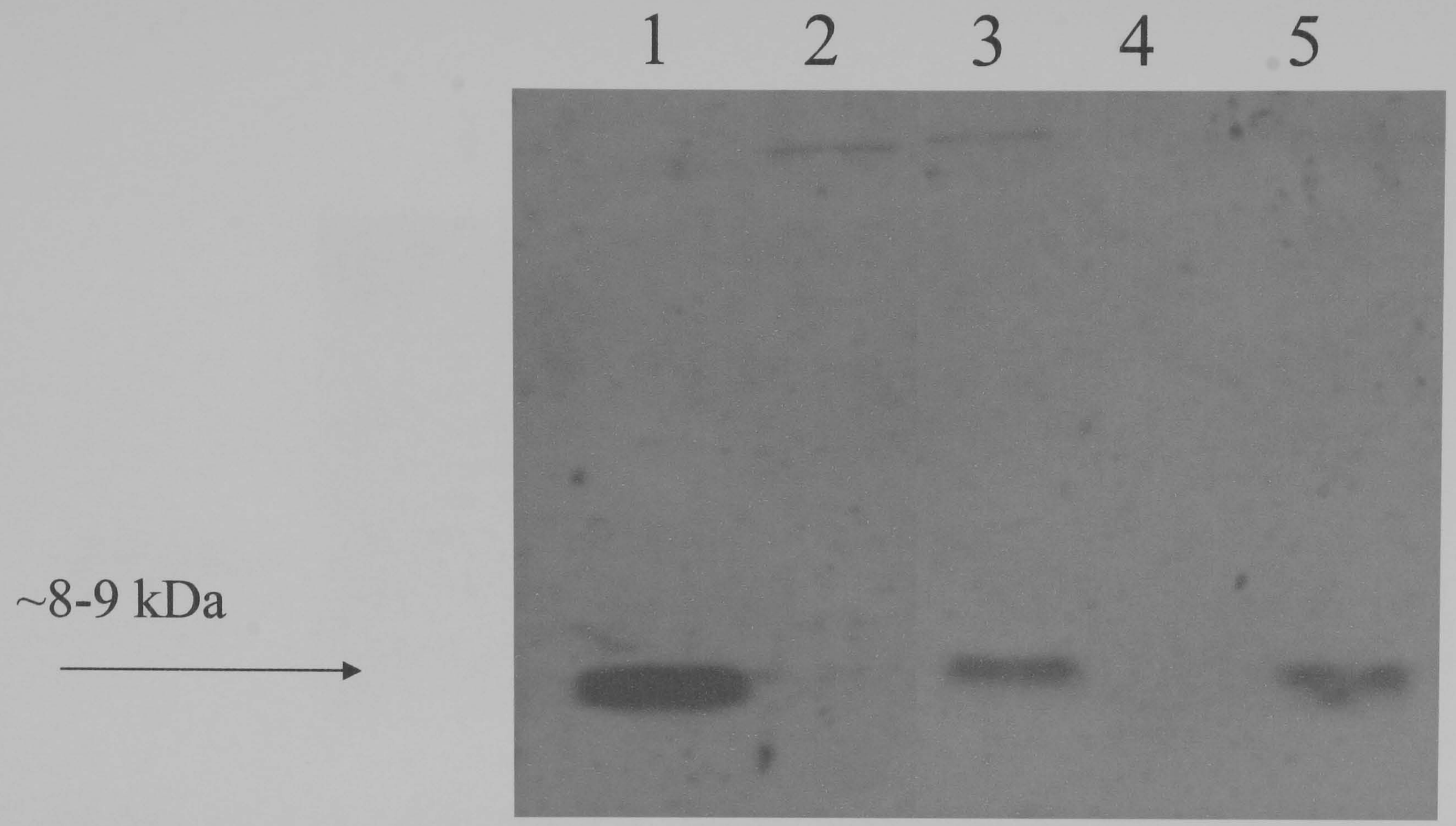


Figure 5.2.5, **Western blot demonstrating murine CCL20 expressed by HEK293 transfected with pcDNA3.1-mCCL20 and visualised with polyclonal antisera to rat CCL20.** Semi-confluent HEK293 cells were transfected with 10μg of cloned pcDNA-murineCCL20 or media only. Supernatants were harvested 72 hours post-transfection, spepearted on a 15% polyaccrulamide gel and assayed by Western blotting. Expressed protein migrated with an apparent size of 8-9 kDa

.**Lane 1**= 25 ng recombinant rat CCL20, **Lane 2**= supernatant from media control transfection #1, **Lane 3**= supernatant from cells transfected with pcDNA-CCL20 , transfection #1 **Lane 4**= supernatant from media control transfection #2, **Lane 5** = supernatant from cells transfected with pcDNA-CCL20 transfection #2

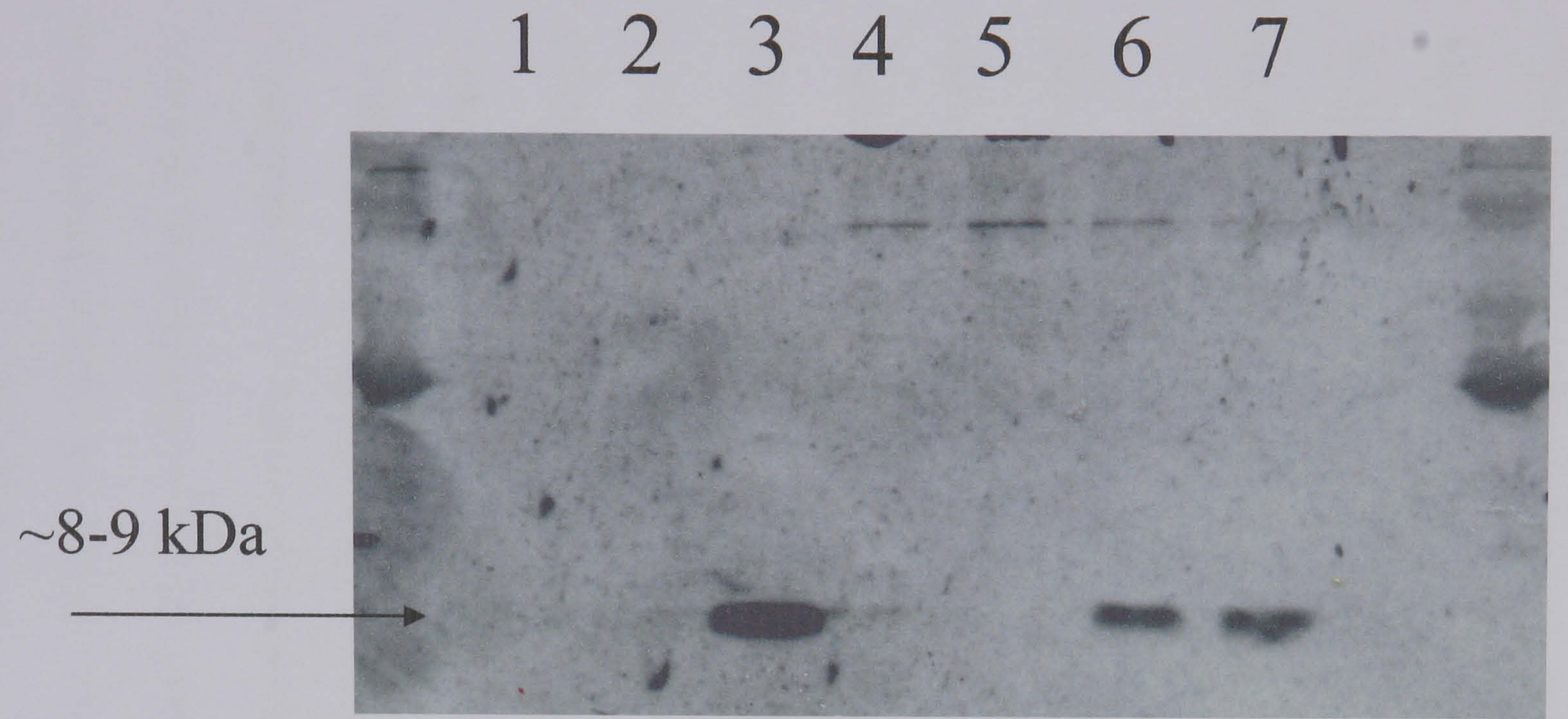


***Figure 5.2.6*, Western blot demonstrating murine CCL20 expressed by HEK293 transfected with pcDNA3.1-mCCL20 and visualised with monoclonal antisera to murine CCL20.** Semi-confluent HEK293 cells were transfected with 10μg of cloned pcDNA-murineCCL20 or media only. Supernatants were harvested 72 hours post-transfection, separated on a 10% polyacrylamide gel and assayed by western blotting by probing for one hour with a biotinylated monoclonal anti-mouse CCL20 antibody followed by a streptavidin horse radish peroxidase conjugate. The blot was exposed for two minutes after treatment with ECL reagent (Amersham). Expressed protein migrated with an apparent size of 8-9 kDa.
Lane 1= 25 ng recombinant rat CCL20, **Lane 2**= 25ng recombinant human CCL20, **Lane 3**= 25ng recombinant murine CCL20, **Lane 4**= supernatant from cells transfected with an empty vector #1, **Lane 5** = supernatant from cells transfected with an empty vector #2., **Lane 6**= supernatant from cells transfected with pcDNA-CCL20 #1, **Lane 7**= supernatant from cells transfected with pcDNA-CCL20 #2.

5.2.3 Verification of cloned CCL20 biological activity

5.2.3.1 Calcium Flux Assay

CCL20 has previously been shown to induce calcium flux in CCR6 positive cells (Baba *et al.* 1997). To confirm the biological activity of cloned CCL20, supernatants from HEK293T cells, transiently transfected for 48 hours with pcDNA3.1-CCL20 or recombinant rat CCL20, used to stimulate rat splenocytes in a calcium flux assay (with the aid of Dr James Peace, Imperial College). Rat splenocytes were harvested and T cells and B cells purified by passage of single cell suspension through nylon wool columns.

200nM or 500nM of recombinant rat CCL20 or 10nM of SDF-1α were used for positive controls of calcium flux (figure 5.2.7). From the experiments performed, rat splenocytes did not flux in response to CCL20, or the control chemokine SDF-1α. However, the Ca^{2+} release assay was still valid, as the detergent Triton-80 results in a flux. To address this issue in a more specific manner, cell lines stably transfected with human and murine CCR6 were examined for calcium flux with recombinant human and murine CCL20 respectively. Non-transfected HEK293T cells were used as a negative control (figure 5.2.8). Upon addition of either the CCL20 obtained from transfected HEK293 cells, or recombinant CCL20 (arrows and labels), there was a small species specific calcium flux. However, the magnitude of the flux generated was not significantly above background to be considered positive. In a personal correspondence with Dr James Peace, cell lines can be difficult to generate a calcium flux.

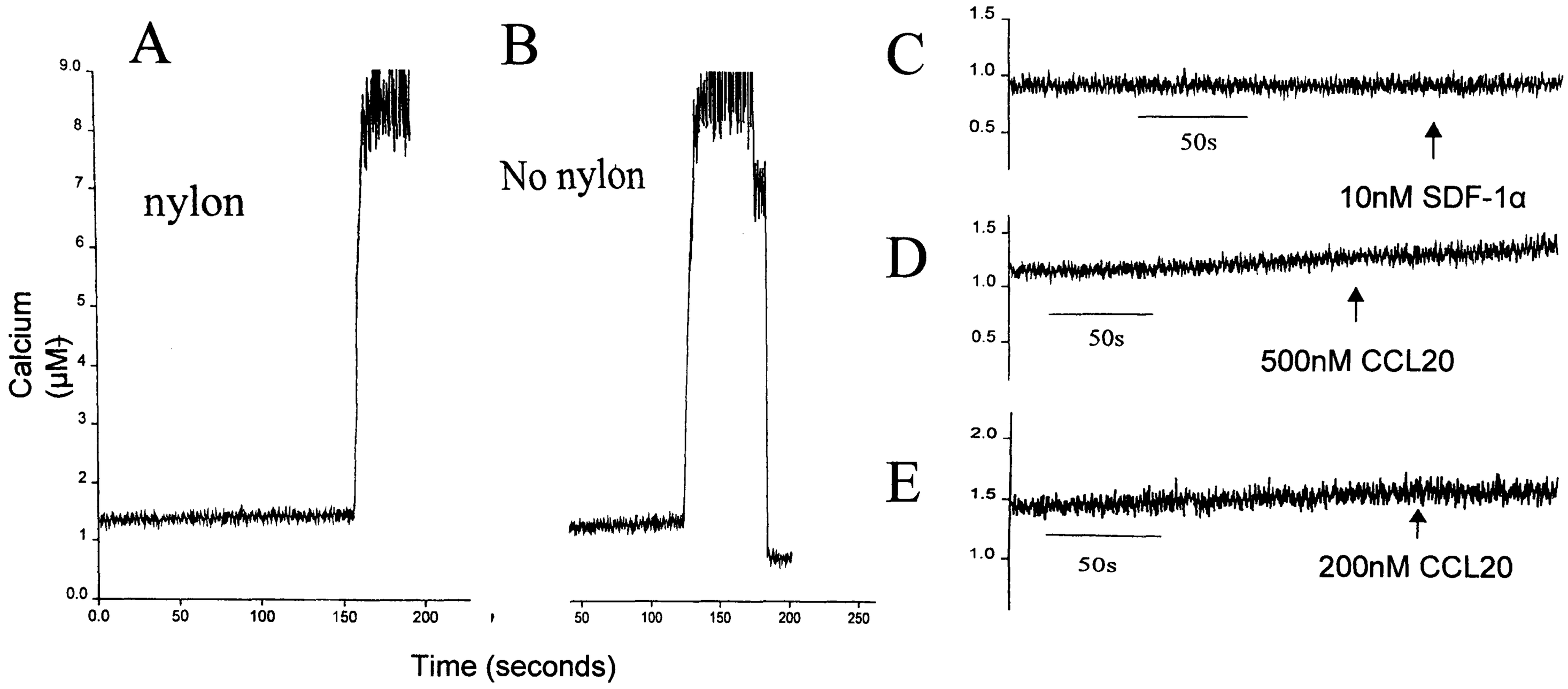
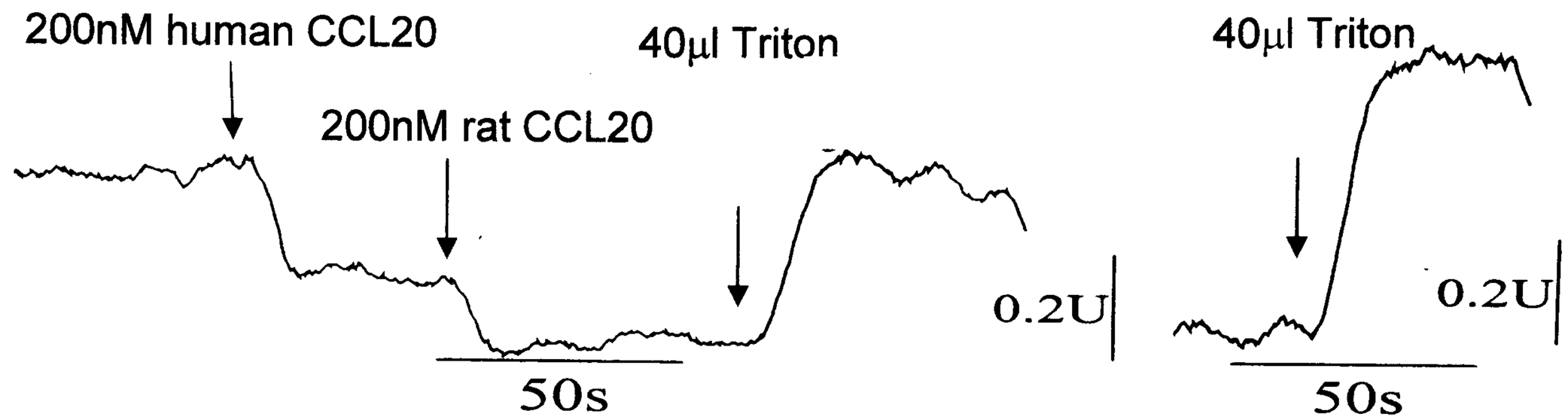
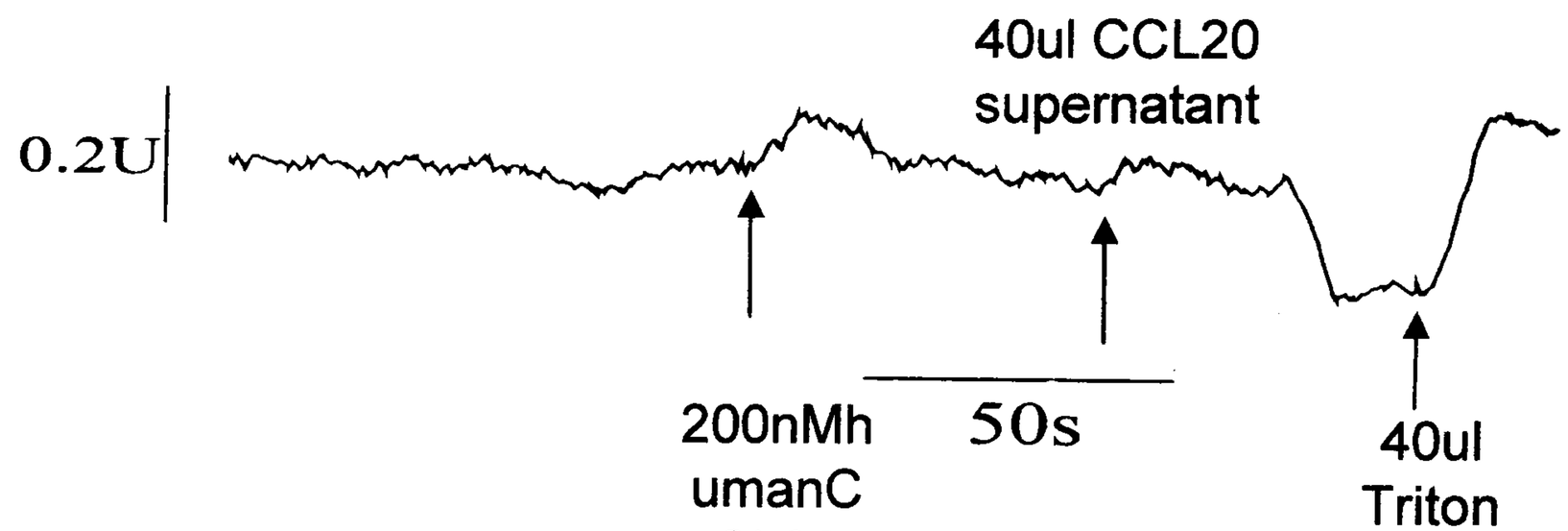


***Figure 5.2.6*, Calcium flux of rat splenocytes in response to chemokines.** Rat splenocytes were examined for their ability to induce a calcium flux in response to treatment with recombinant rat CCL20 (D & E). The chemokine human SDF-1α (C) was used as a positive control. Two methods of purification were tested, either passing the splenocytes through a nylon cloth or not (A&B), and examining the ability to flux in response to the detergent Triton.

Control HEK293



huCCR6 HEK293



muCCR6 HEK 293

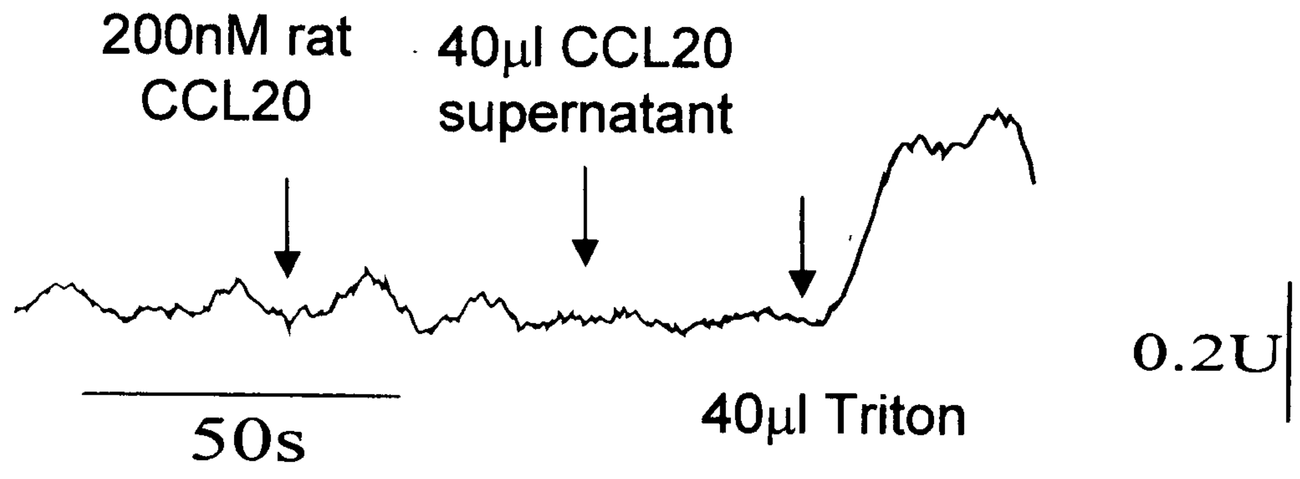


Figure 5.2.8, HEK 293 cell lines, stabley expressing either human CCR6 or the murine CCR6, were examined for their ability to calcium flux when treated with recombinant CCL20 or supernatants from plasmid expressed CCL20. As a positive control, the detergent Triton was used to induce a flux.

5.2.3.2 Transmigration Assay

5.2.3.2a Validation of CCR6 expression by bone marrow derived DCs.

CCL20 was reported to promote migration of CCR6 positive cells in a concentration dependent manner (Charbonnier *et al.* 1999). In order to validate the capacity of bone marrow derived DCs to respond to CCL20 in a transmigration assay, CCR6 expression was confirmed by flow cytometry. Bone marrow derived dendritic cells (BMDCs) were cultured in the presence of GM-CSF, and examined by flow cytometry for CD11c (a murine DC cell marker), MHC class II and CCR6 expression. 59% of the gated population co-expressed $CD11c^{+}$, MHC class II^{+} and $CCR6^{+}$ (Figure 5.2.9).

5.2.3.2b Transwell migration assay.

The chemotactic potential of recombinant and transfected CCL20 were determined by assessment of their respective capacity to cause $CCR6^{+}$ BMDCs to migrate from the upper to the chemokine containing lower chamber in a transwell migration assay. The effects of a range of recombinant murine CCL20 concentrations and supernatants from HEK293T cells transfected with the CCL20 plasmid or supernatants from control transfected cell were compared for their capacity to induce migration. Cells that had migrated into the lower chamber or onto the lower side of the filter were counted after 4 hours. The results showed that there was no significant difference between wells containing the chemokine CCL20 and control wells. Therefore, in this assay bone marrow derived dendritic cells do not migrate in a concentration dependent manner towards CCL20 (Figure 5.2.10).

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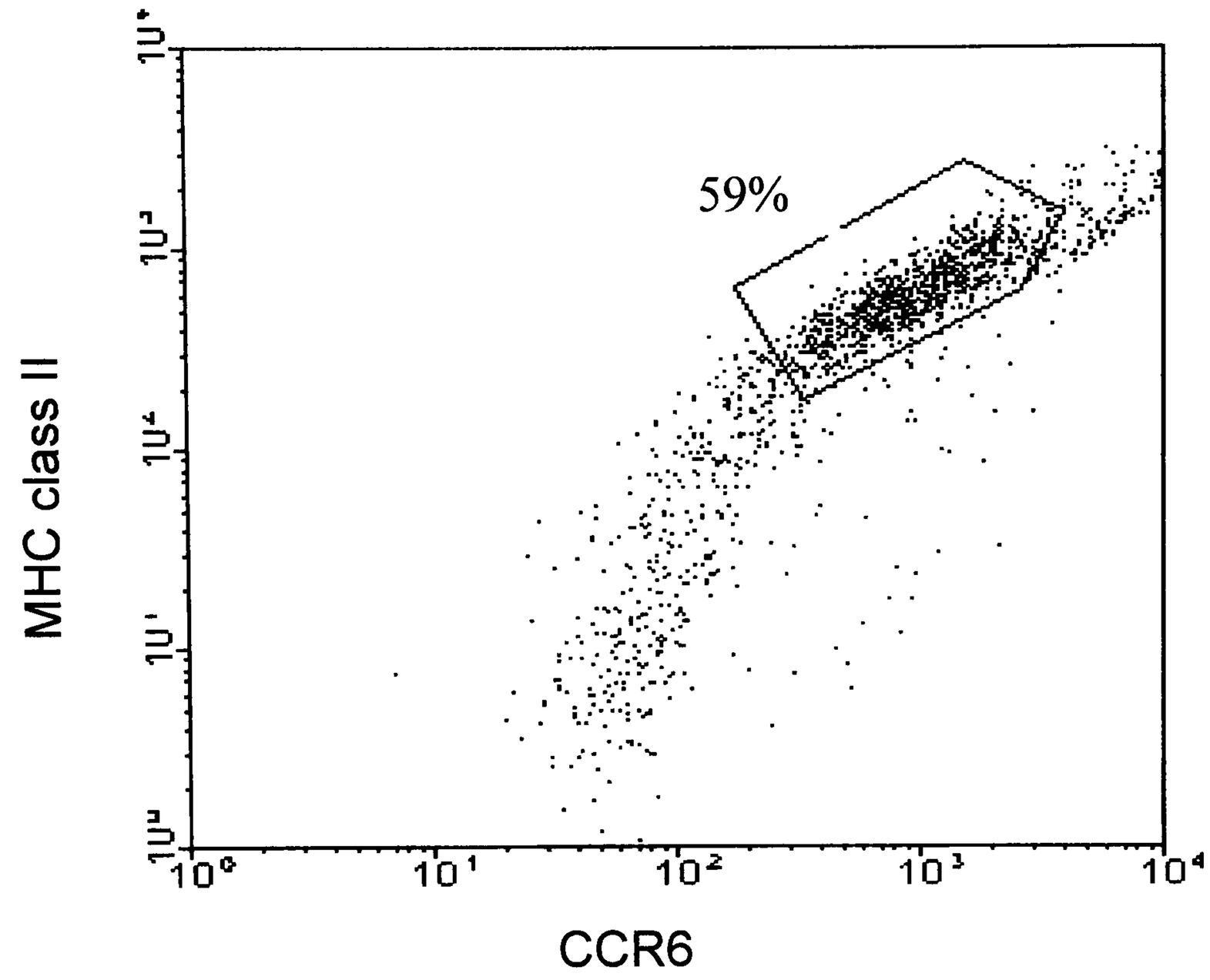

***Figure 5.2.9*, Flow cytometric analysis of bone marrow derived DCs**. Cells were harvest from bone marrow from balb-c mice, and cultured for 6 days in the presence of GM-CSF (20ng/ml). Cells were purified using Milltenyi CD11c beads, gated for live cells based on the FSC vs SSC, and examined for the presence of MHC class II and CCR6.

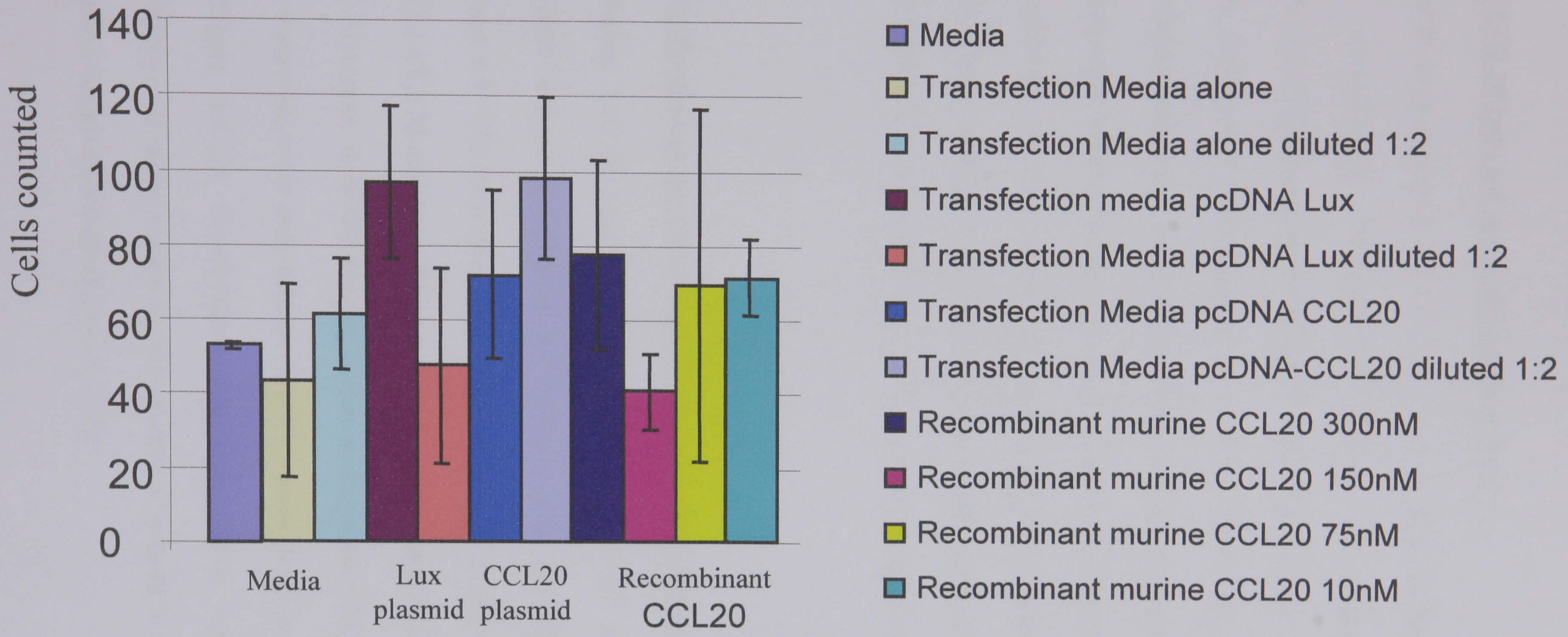

***Figure 5.2.9*, Transmigration of Bone marrow derived derived DCs in response to CCL20.** Bone marrow cells were cultured in GM-CSF for 6 days, and tested for their ability to migrate to CCL20. Variables tested are media alone, Media from a null transfection, Media from an irrelevant plasmid, and media from a CCL20 expression plasmid. Migration responses were compared to those observed with known concentrations of recombinant Results are the mean of 5 replicates with error bars of the standard deviation

5.2.3.3 CCL20 induced tyrosine phosphorylation.

Another biological property of CCL20 is the induction of tyrosine phosphorylation upon CCR6 cross-linking. Recombinant CCL20 and supernatants from 48-hour pcDNA3.1-CCL20 transfected HEK293T cells were used to stimulate BMDCs for five minutes. Cell lysates were resolved on a 10% polyacrylamide gel and transferred to a nitrocellulose membrane for Western blotting. Upon development of blot with an anti-phosphotyrosine HRP antibody, both recombinant CCL20 and tissue culture supernatants from pcDNA3.1-CCL20 transfected cells, induced tyrosine phosphorylation within BMDCs. These bands were not evident in the media alone or mock transfected controls (Figure 5.2.11). This shows that CCL20 is inducing a signal cascade, and is biologically active.

5.2.4 Verification of recombinant CCL20 expression *in vivo*.

Recombinant CCL20 expression *in vivo* was confirmed by RT-CPR. In order to differentiate between recombinant and endogenous CCL20 mRNA, we utilised a 5' T7 primer and a 3' CCL20 primer. The T7 promoter is 3' to the CMV promoter within the pcDNA3.1-CCL20 construct. Therefore, it is an integral part of the recombinant CCL20 mRNA. However, it is not present within endogenous CCL20 mRNA. Recombinant CCL20 transcripts were only present in cDNA preps from pcDN3.1-CCL20 inoculated mice (Figure 5.2.12). Confirmation that these bands were not due to plasmid contamination was demonstrated by the absence of a band in the corresponding reverse transcription negative controls (Figure 5.2.12).

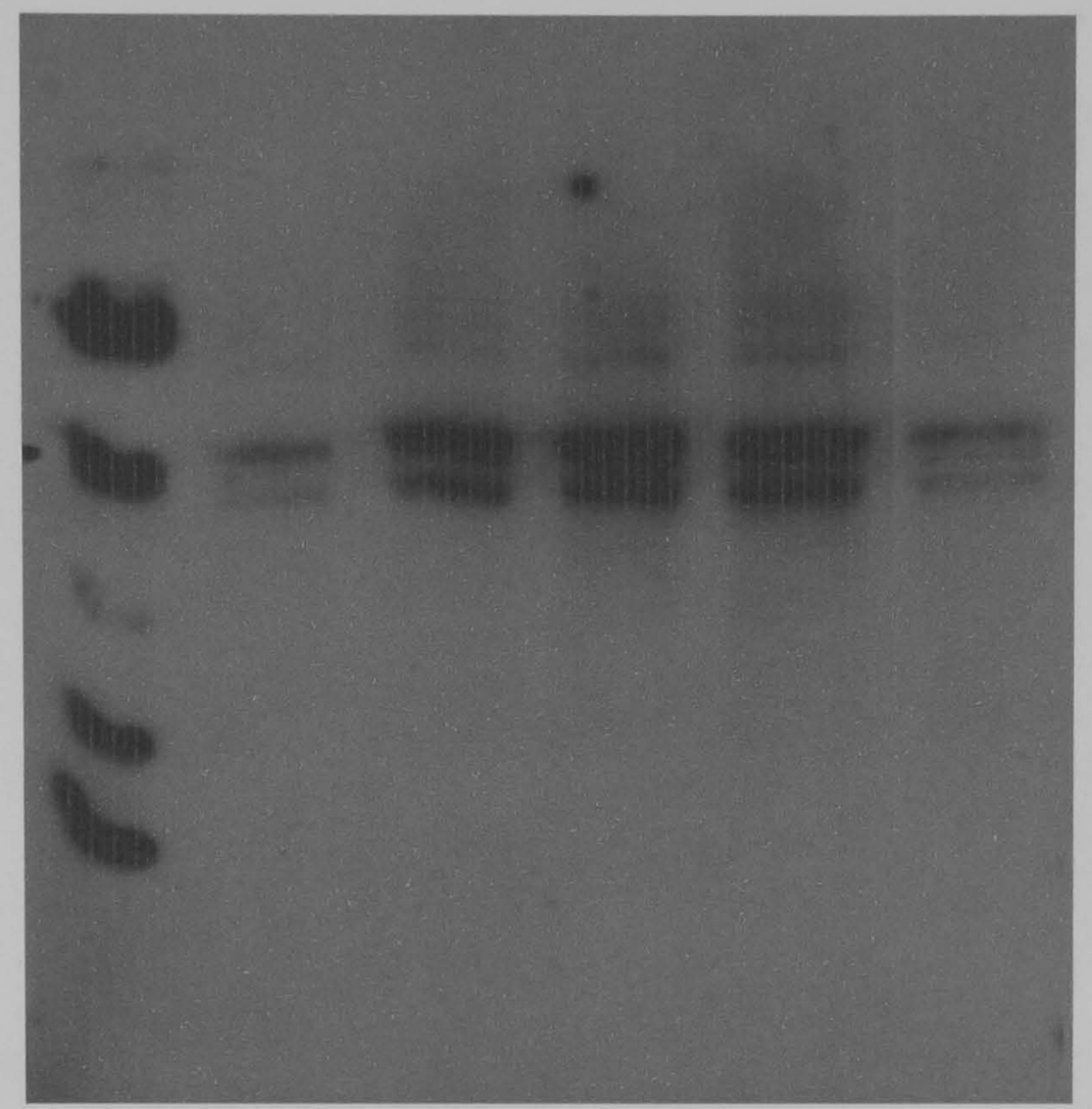

Figure 5.2.10, **Supernatant from pcDNA-MIP-3α transfected HEK-293 cells stimulate tyrosine phosphorylation and activation of the MAPK pathway in bone marrow derived dendritic cells.** *1x 10^6 bone marrow derived cells were treated for 5 minutes at 37° C with the appropriate stimulant and then lysed. Cell extracts were separated on a denaturing protein gel which, after transfer onto a nylon membrane, were probed for two hours with MoAb. to human phospho-ERK, followed by 1 hour incubation with a horse radish peroxidase conjugated anti-mouse IgG antibody. The filter was treated with ECL reagent (Amersham) and then exposed for 30 seconds. Both bands visualised in the blot, demonstrate the phosphorylation of tyrosine.*

***M**= protein marker, **Lane 1**= media control, **Lane 2**= recombinant murine CCL20 (100nm), **Lane 3**= recombinant CCL20 (10nm), **Lane 4**= supernatant derived from pcDNA-CCL20 transfected HEK cells, **Lane 5**= supernatant derived from media only transfected HEK cells*

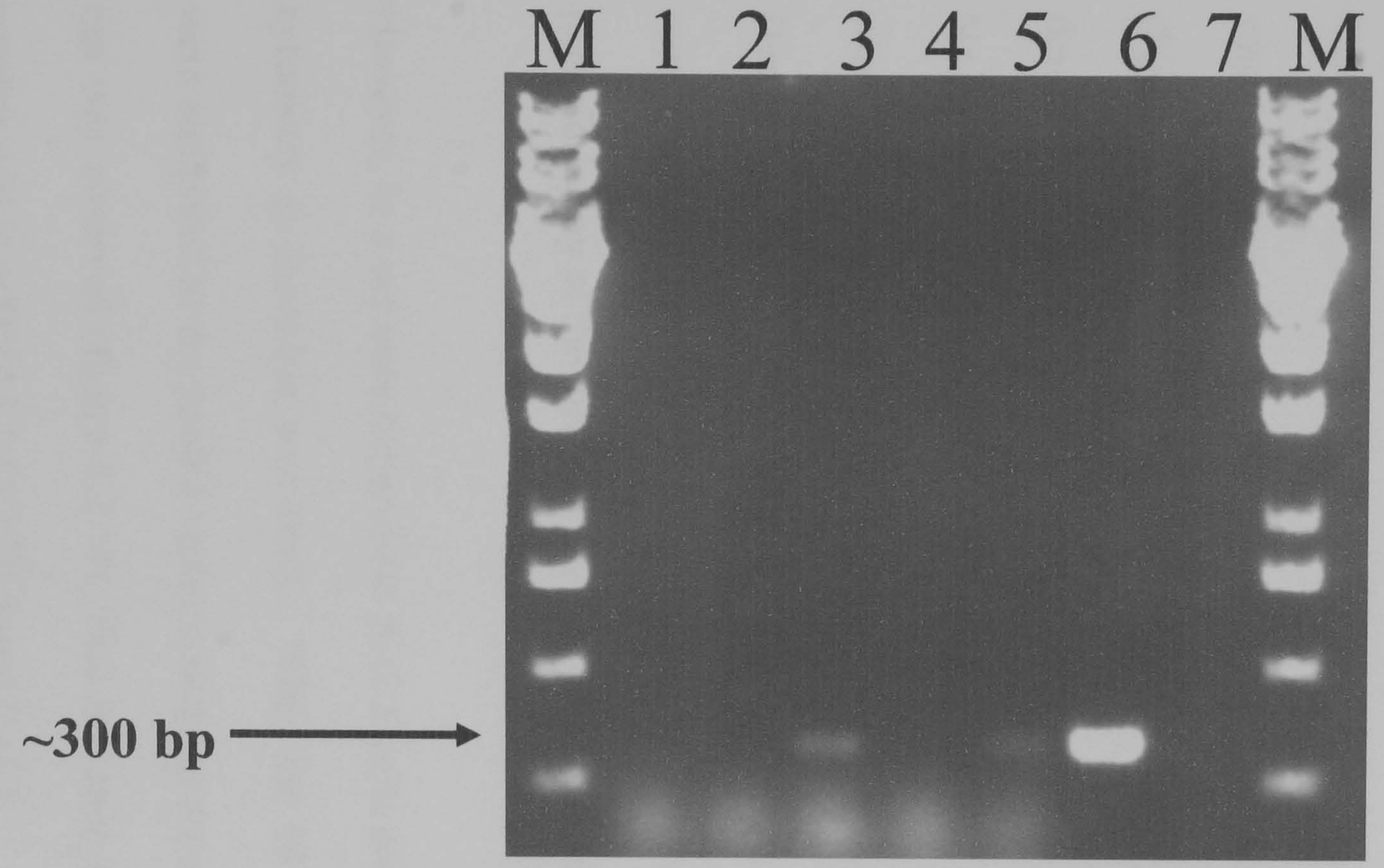

***Figure 5.2.12*, CCL20 mRNA from NALT transfected with a plasmid pcDNA3.1-CCL20.** At 24 and 48 hrs. following intranasal administration of cytofectin-complexed pDNA encoding CCL20 or the empty vector, nasal tissue (pooled from six mice per group) was isolated, homogenised and mRNA was extracted using Trizol and QIAGEN RNAeasy columns. 10μg of mRNA was used to synthesise cDNA. Murine CCL20 cDNA was amplified using T7 (forward) and CCL20 internal (reverse) primers to demonstrate expression of plasmid derived CCL20 from low level constitutively expressed CCL20.

M= 1 kb marker, **Lane 1**= sample from naïve mouse , **Lane 2**= sample isolated 24 hr. following immunisation with the empty control vector, **Lane 3**= sample isolated 24 hr. following immunisation with pcDNA-MIP-3α, **Lane 4**= sample isolated 48hr. following immunisation with the empty control vector, **Lane 5**= 48 hr. mouse immunised with pcDNA-CCL20, **Lane 6**= 1 ng plasmid DNA (pcDNA-CCL20) PCR positive control, **Lane 7**= PCR negative control (water).

5.2.5 Recruitment of DCs following intranasal administration of pcDNA3.1-CCL20.

After demonstration of the presence of recombinant CCL20 transcripts at the site of inoculation, it was critical to then demonstrate the functional activity of the recombinant CCL20. This was determined by flow cytometric analysis of DC number within inoculated versus naive NALT. Mice were inoculated with the plasmid encoding CCL20, via the intranasal route. At 48 and 72 hours NALT was harvested and DCs defined by CD11c, CD11b and MHC class II expression (flow cytometry). The percentage of DCs was calculated as the proportion of the total cell number for each individual NALT (Figure 5.2.13). Each individual graph represents a separate experiment. At the 48 hour time point there is a trend towards increased DC number in pcDNA3.1-CCL20 inoculated NALT. However the difference between dosed and control mice was not significant and when the experiment was repeated with a larger group size, no such trend was observed. Due to the technical difficulties of harvest NALT, a large degree of variability can be seen, resulting in no significant difference between the vector control and the CCL20 plasmid in any of the different experiments either at 72 or 48 hours.

However, in a separate experiment NALT cells preps were pooled and analysed by flow cytometry as described previously. When the 48 hour pcDNA3.1-CCL20 group (n=5) were compared to the pooled vector control group (n=5), a 2 fold increase in DC number can was observed (figure 5.2.14). This indicates that CCL20 expression increased DC number within pcDNA3.1-CCL20 dosed NALT.

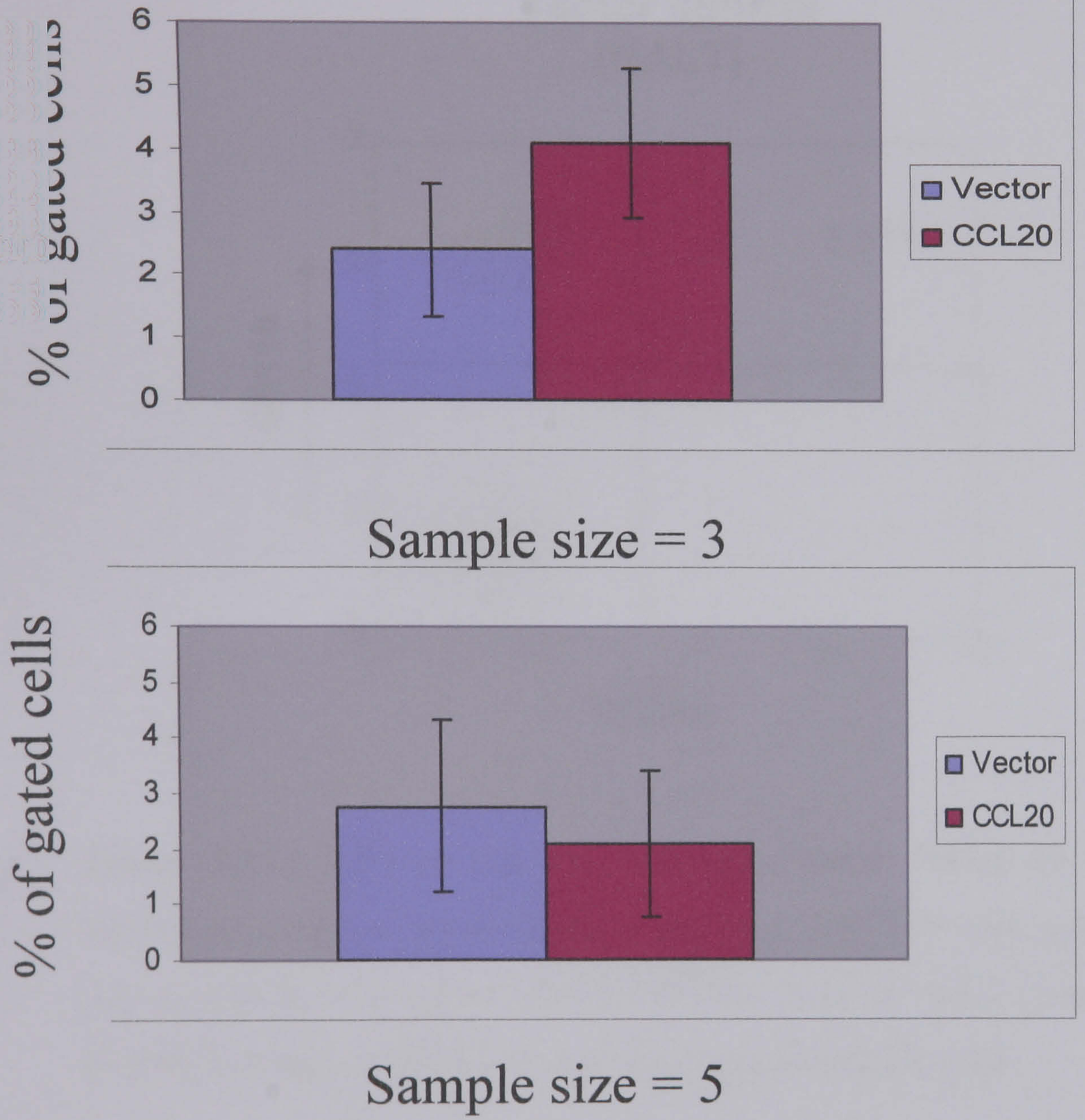

***Figure 5.2.12*, Analysis of DC populations in nasal epithelium at 48 and 72 hours, following I/N administration of pcDNA3.1-CCL20.** $CD11c^+$ $CD11b^+$ cell populations isolated from NALT, 48 or 72 hours post intranasal immunisation with a CCL20 expressing plasmid or an empty vector. Each graph represents a separate experiment, and each mouse was analysed individually. Results are the mean of 5 replicates with error bars of the standard deviation

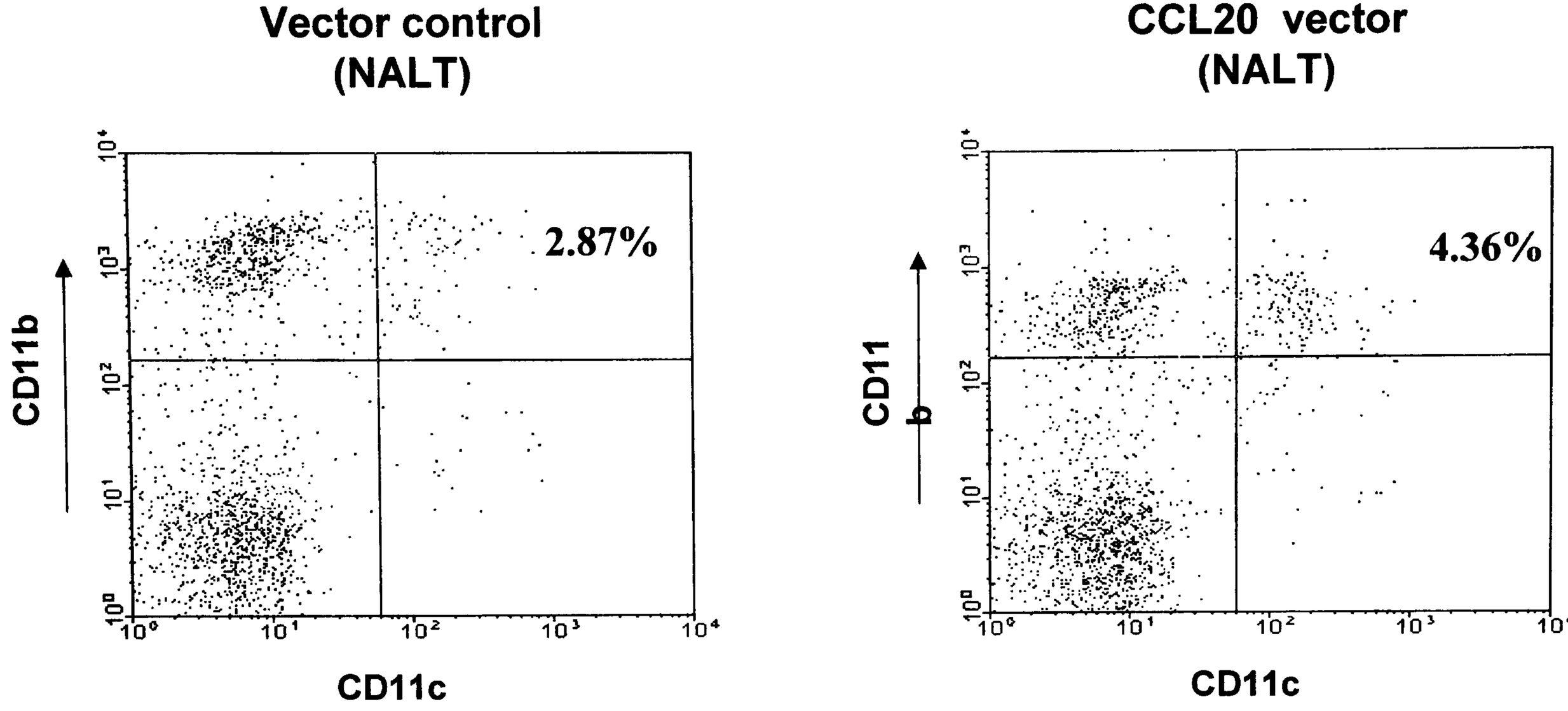

***Figure 5.2.13*, CD11c+ CD11b+ cells recruited to NALT 48 hours after IN inoculation of a CCL20 expressing plasmid**. 48 hours post intranasal immunisation with either a CCL20 expressing plasmid or the empty vector, NALT from 5 mice was extracted and pooled, and stained for CD11c and CD11b. In the vector control 2.87% of the population were CD11c+ and CD11b+ as opposed to 4.38% of the population in the CCL20 expressing plasmid.

5.2.6 Migration of MHC class II+ cells upon subcutaneous administration of pcDNA3.1-CCL20.

CCL20 has been reported to induce the migration of Langerhan cells to the skin (Dieu-Nosjean *et al.* 2000). By administration of CCL20 encoding plasmid subcutaneously, it was hypothesised that DC frequency should increase. This hypothesis was tested by the administration of 50μg of pcDNA3.1-CCL20 or control vector, subcutaneously into the base of the tail. 24, 36, 48, and 72 hours later the tails were harvested and analysed for DC density by immunohistochemistry (MHC class II^{+ve} cells).

A typical section of tail tissue stained for MHC class II is illustrated in figure 5.2.15, MHC class II+ cells are highlighted by arrows and were counted. At least three sections were taken per animal and from each section 5 field of views were blind counted. After 24 hours and 36 hours, both CCL20 plasmid and vector control induced an increase in MHC class II^{+ve} cells frequency. However, at 48 hours the pcDNA3.1-CCL20 dosed skin has higher DC numbers than the vector control, suggesting plasmid encoded CCL20 in situ is recruiting MHC class II positive cells. However, after 72 hours, the CCL20 dosed skin has a frequency of MHC class II^{+ve} cells equivalent to that found in the vector control dosed skin.

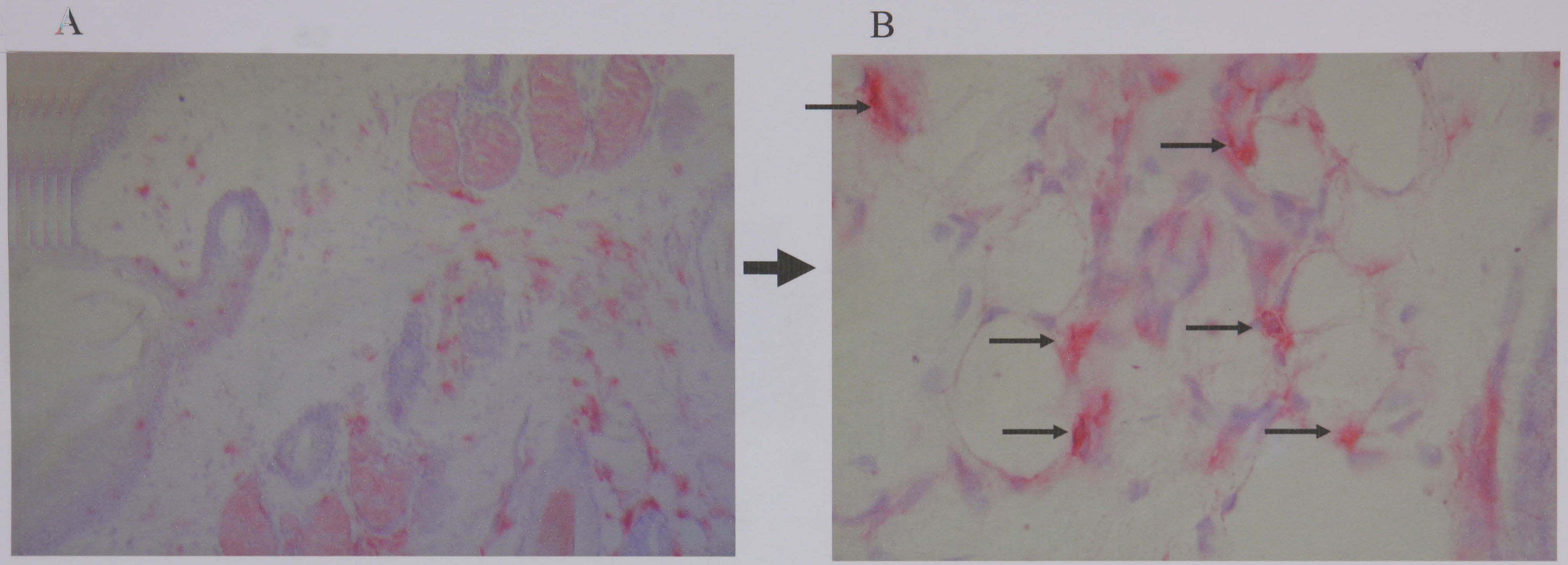

***Figure 5.2.14*, Sections stained for MHC class II after subcutaneous administration of plasmid DNA expressing CCL20.** Immunohistochemistry of murine tail sections stained for MHC class II and counterstained with haematoxylin. Panel A is a typical field of view at x20 magnification, and (B) indicates MHC class II^{+ve} cells at a x100 magnification. From the morphology, these cells were identified as dermal DCs (R.Tigler, personal communication).

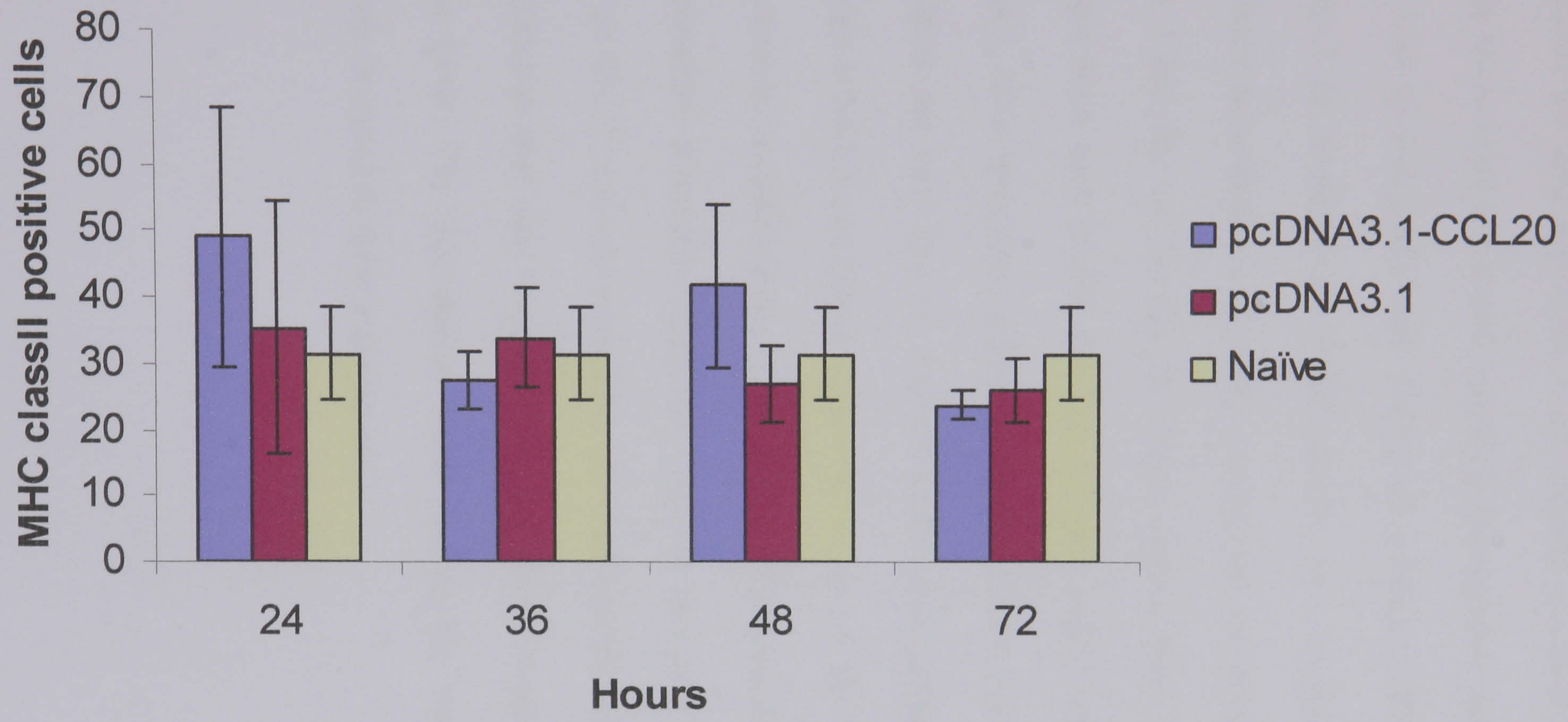

Figure 5.2.15, **Recruitment of MHC class II positive cells upon subcutaneous immunisation of a CCL20 expressing plasmid.** Mice were immunised with 50μg CCL20 expressing plasmid into the base of the tail. At 24, 36, 48, or 72 hours following injection, tails were harvested and sectioned. Sections were stained for MHC class II, and positive cells were blind counted. Each section had 3 fields of views counted, and each mouse had 3 sections counted. Results are the mean of 3 replicates with error bars of the standard deviation

5.2.7 Co-inoculation of pcDNA3.1-CCL20 with VR-SEAP.

To examine the adjuvant effect of the plasmid encoded CCL20, pcDNA3.1-CCL20 was co-immunised with a plasmid encoding the reporter gene SEAP, VR-SEAP. 50μg of SEAP was co-inoculated with 100μg of pcDNA3.1-CCL20 via the intranasal route. pcDNA3.1 or recombinant SEAP protein with cholera toxin (CT) were inoculated intranasally as controls. 5 mice were immunised per group, and each group were boosted on day 7 and day 14. On day 28, blood, vaginal fluid, NALT, CLN and spleens were harvested from each mouse and the antibody and T cell proliferation responses were examined. Mice inoculated with recombinant SEAP and CT demonstrated induction of IgG within the sera, IgA in vaginal washes and antigen-specific T cell proliferative responses in NALT and spleen (Figure 5.2.17 to 5.2.19). Plasmid DNA encoding SEAP, either alone or co-administered with plasmid DNA encoding CCL20 unexpectedly failed to demonstrate detectable serum IgG, vaginal IgA or T cell proliferative responses in spleen or NALT. Gel electrophoresis of VR-SEAP plasmid DNA indicated a high level of degradation that was not present in the initial investigation into the quality of the plasmid DNA. The degradation could explain the reason for the lack of immune responses observed in these experiments.

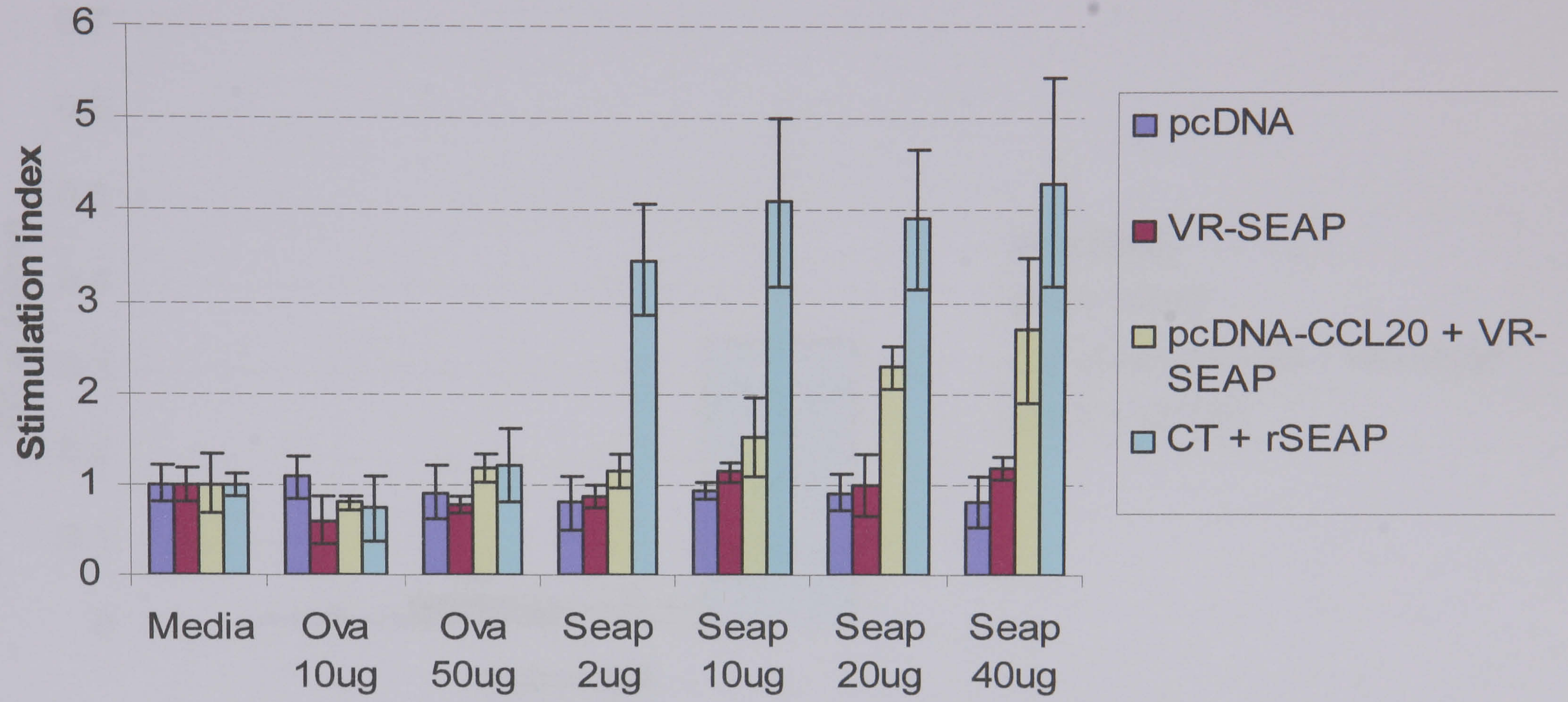

Figure 5.2.17, **T cell proliferative responses of mice immunised with plasmid DNA encoding CCL20 and SEAP.** Mice were co-immunised with 100μg plasmid DNA encoding CCL20 and 50ug plasmid DNA encoding SEAP intranasally and boosted at day 7 and 14. On day 28, splenocytes were incubated with recombinant SEAP or Ova (as control) and pulsed with tritiated thymidine. The data represents the mean and and standard deviation calculated from 8 replicates.

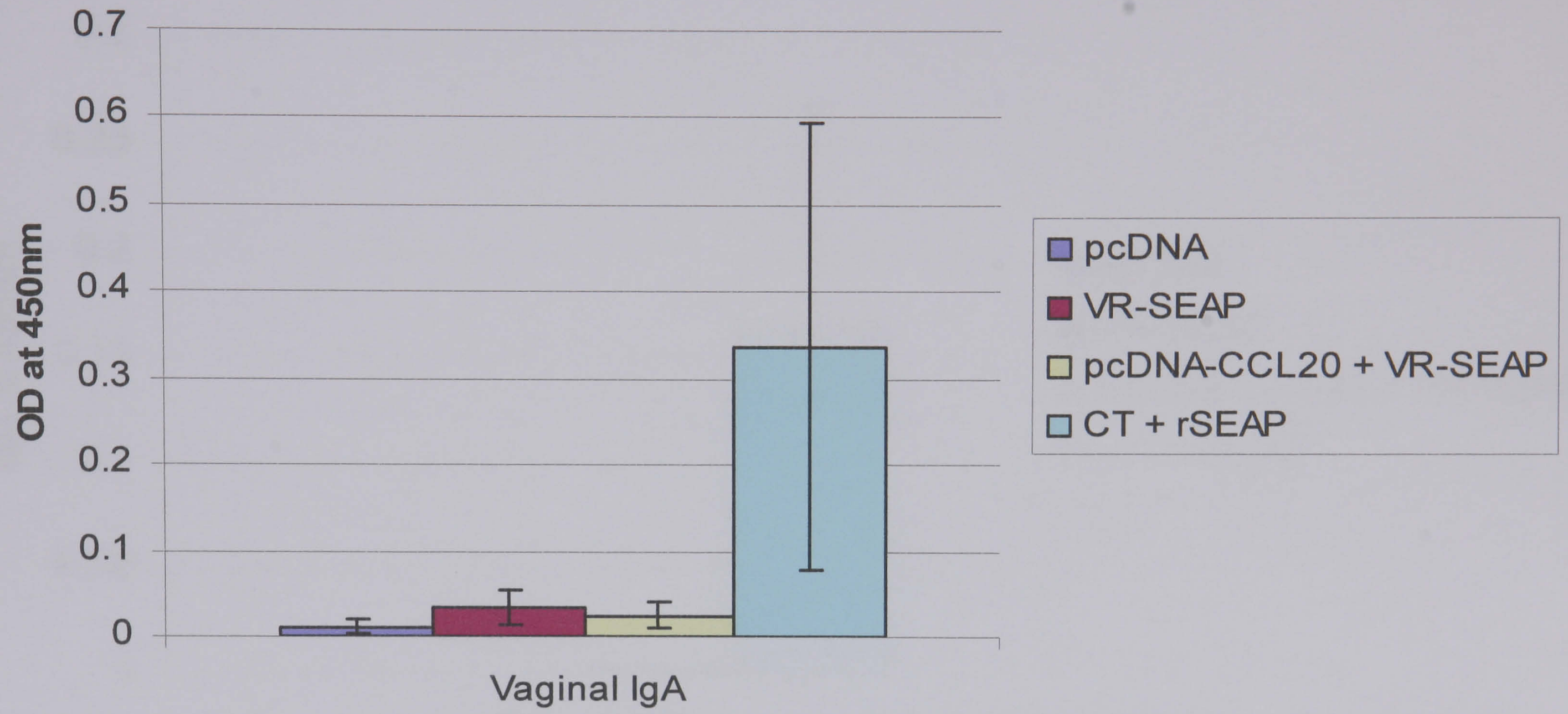

***Figure 5.2.18*, SEAP specific IgA detected following intranasal immunisation.** Mice were immunised by the intranasal route with 100μg plasmid DNA encoding CCL20 and 50ug plasmid DNA encoding SEAP and boosted at day 7 and 14. At day 26 vaginal washes were collected, diluted 1 in 2 and examined for IgA specific for recombinant SEAP. The data represents the mean and and standard deviation calculated from 8 replicates.

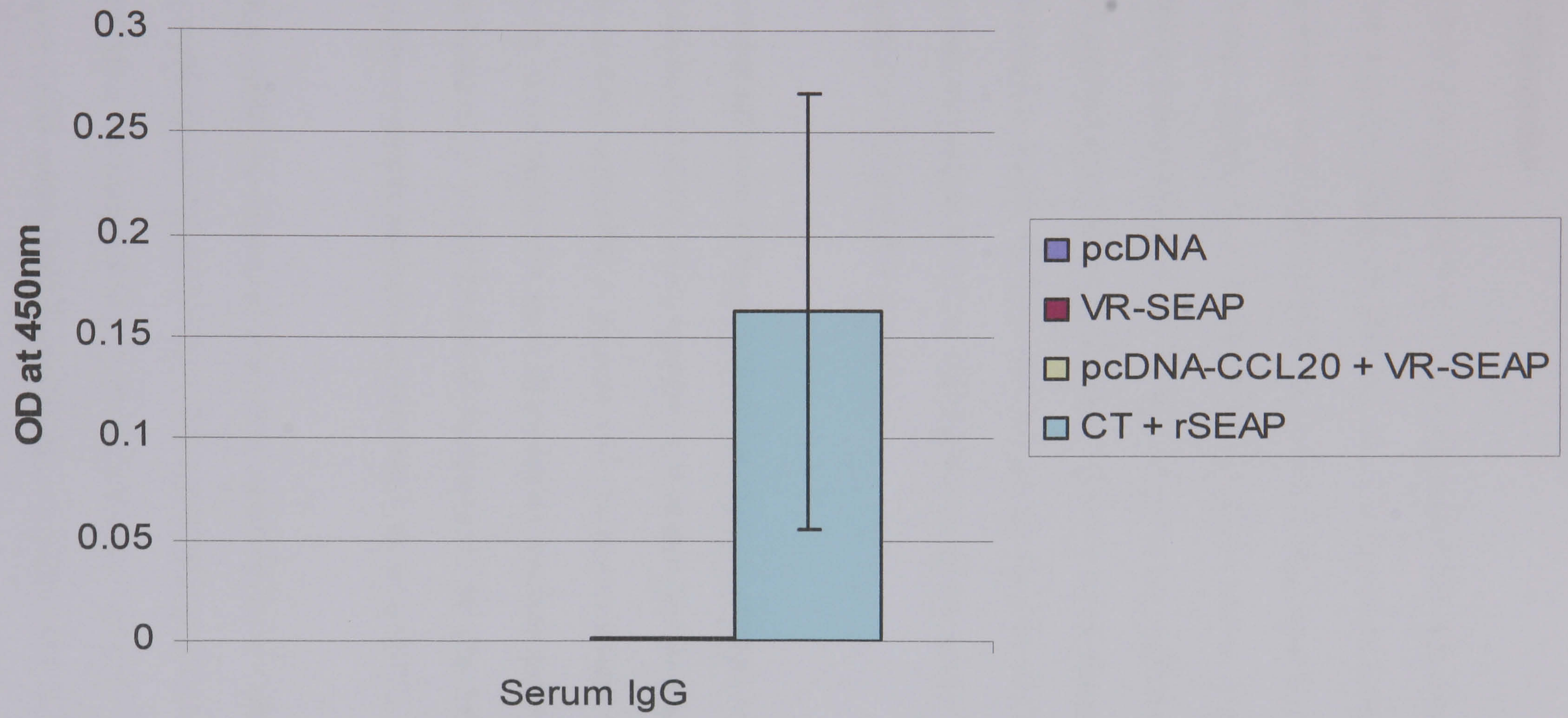

***Figure 5.2.19,* SEAP specific IgG detected following intranasal immunisation.** Mice were immunised by the intranasal route with 100μg plasmid DNA encoding CCL20 and 50ug plasmid DNA encoding SEAP and boosted at day 7 and 14. At day 26 vaginal washes were collected, diluted 1 in 2 and examined for IgG specific for recombinant SEAP. The data represents the mean and standard deviation calculated from 8 replicates.

5.3 Discussion

The biological properties of CCL20 suggested that this chemokine may be suitable for use as a genetic adjuvant. This was due to the observations that CCL20 was a potent chemoattractant for DCs, which are primary APCs capable of producing strong immune responses (Akbari *et al.* 1999). CCL20 was also shown to be upregulated within both epithelia (Dieu-Nosjean et al, 2000) and the mucosa (Power et al, 1997) after antigen challenge/infection. It was hypothesised that the over-expression of CCL20 at the site of immunisation would increase DC number at the site of immunisation and therefore increases the magnitude of the subsequent immune response. This may greatly enhance the efficacy of plasmid DNA vaccines.

The intranasal route of immunisation has many advantages over parenteral routes; these include ease of administration, patient compliance and no requirement for injection. This route is more acceptable to patients and also shows greater efficacy than oral or rectal delivery in its capacity to generate combined mucosal and systemic immune responses (Klavinskis *et al.* 1999). Intranasal immunisation has also been shown to be capable of induction of cellular and humoral immunity (Gao *et al.* 1997).

Studies within the respiratory tract have shown CCL20 is intrinsic to the recruitment of DCs to the trachea (Stumbles *et al.* 2001), and other mucosal compartments (Cook *et al.* 2000). Here we have confirmed the expression of endogenous CCL20 and its cognate receptor CCR6 within NALT, supporting a biological role for CCL20 in the recruitment of CCR6 positive cells to NALT.

CCL20 was cloned into an expression plasmid and production of the encoded protein confirmed by *in vitro* translation and western blot analysis. Although anti-murine CCL20 was not available, we have shown that anti-rat CCL20 cross-reacts with murine CCL20 (5.2.5). We were therefore able to use anti-rat CCL20 to prove expression of plasmid encoded murine CCL20. We then attempted to confirm the biological activity of the plasmid encoded CCL20 by calcium flux, transmigration assay and intracellular signalling (tyrosine phosphorylation).

It has been reported that CCL20 can induce calcium flux within CCR6 positive cells (Baba *et al.* 1997). Therefore to validate the biological activity of the plasmid encoded CCL20 we investigated its potential to induce calcium flux in rat splenocytes. We compared the capacity of tissue culture supernatants from pcDNA3.1-CCL20 or control transfected HEK293T cells to induce calcium flux with that of recombinant CCL20 (rat) and SDF-1α, which have been shown to induce calcium flux (Delgado *et al.* 1998). Due to the lack of murine CCL20 at this time, the experiment had to be performed with rat splenocytes as recombinant rat CCL20 was available. It was theorised that the rat splenocytes would express CCR6, as mRNA for CCR6 has previously been identified within murine spleen (Varona *et al.* 1998). Also, no antisera to rat CCR6 was available at this time. Therefore, we could not confirm the presence of CCR6 protein on these cells.

Although a calcium flux was not induced by either recombinant or plasmid encoded CCL20, the positive control, SDF-1α, also failed to induce calcium flux. However, a

calcium flux could be induced by the detergent Triton. The simplest explanation to the failure of this experiment was that the cells were non-viable at the time of this analysis. However, it may also due to our inability to ensure the cells within a field of view were cells $CCR6^{+ve}$, due to a lack of available anti-CCR6 antibody at the time of the experiment. To examine if CCL20 could induce a calcium flux in human or murine $CCR6^{+ve}$ stable cell-lines, these cells were stimulated with the appropriate species of CCL20. Although a calcium flux could be observed upon addition of human CCL20, the magnitude of the response was not significantly above background to be defined as a positive.

Another biological property of CCL20 is to induce $CCR6^{+ve}$ cell migration in a concentration dependant manner (Dieu *et al.* 1998; Charbonnier *et al.* 1999; Yang *et al.* 1999; Dieu-Nosjean *et al.* 2000). Also, bone marrow derived cells have been shown to migrate to CCL20 (Biragyn *et al.* 2001). Therefore, the capacity of recombinant and plasmid encoded CCL20 to attract DCs was assessed. Murine bone marrow derived DCs were cultured in the presence of GM-CSF and their CCR6 status was confirmed by flow cytometry (figure 5.2.10). However, we failed to show an increase in migration under any of the conditions tested. A detailed study of the literature revealed that studies migration of CCR6+ve cells in response to CCL20 utilised human CCL20, no such effect was shown with murine CCL20 (Ogata *et al.* 1999). Our data support these observations.

CCR6 is a G-protein coupled receptor (Zaballos *et al.* 1996; Baba *et al.* 1997), interaction of these receptors with their cognate ligands results in signalling via the Map kinase pathway (Chuang and Ng 1994; Hirano *et al.* 1996) and requires tyrosine phosphorylation. Therefore, to confirm the biological activity of our CCL20 we examined the capacity of plasmid encoded CCL20 versus recombinant CCL20 to induce tyrosine phosphorylation in CCR6 positive cells. Bone marrow derived DCs incubated with either recombinant or plasmid encoded CCL20 exhibited increased levels of tyrosine phosphorylation in comparison to control cultures (Figure 5.2.11). This confirmed the capacity of plasmid encoded CCL20 to signal in a biologically significant manner.

In order to confirm that it is possible to express a plasmid DNA encoded transgene by intranasal inoculation, we examined levels of plasmid encoded CCL20 message by RT-PCR. pcDNA3.1-CCL20 dosed NALT showed CCL20 message levels above those of pcDNA3.1 dosed NALT and normal controls. The validity of this observation was confirmed by the lack of signal in the none reverse transcribed samples. Therefore, it is likely that this message is transcribed and the local levels of CCL20 are elevated. As we have already shown that the expressed CCL20 is biologically active, it is likely that this will result in an increase in the influx of DCs.

To test the hypothesis that local over-expression of CCL20 will increase the efficiency of recruitment of DCs *in vivo*, plasmid DNA encoding CCL20 was inoculated via the intranasal route into balb/c mice. NALT DCs were identified on the basis of CD11c and MHC class II expression. Recruitment of DCs due to recombinant CCL20 expression was

compared to recruitment to empty plasmid DNA. Initial results were inconclusive, with no significant difference between empty plasmid and plasmid encoded CCL20 (Figure 5.2.13). NALT is a very difficult environment to work on in murine systems. Efficient recovery of the complete NALT compartment is troublesome, due to the difficulty of isolating cells from this bony environment. It is likely that this variability cell harvesting obscured any observable biological trend. Also, the number of DCs within NALT, as a proportion of total cells, is very small (less than 1%) making statistically valid cell numbers difficult to obtain from a single mouse. Therefore, we repeated the experiment with pooled NALTs from 5 mice. From the pooled samples we were able to determine a 50% increase in DC number within pcDNA3.1-CCL20 dosed NALT. We also confirmed this observation of increased DC number by immunohistochemical analysis of skin dosed subcutaneously with either pcDNA3.1-CCL20 or empty pcDNA3.1. Slides were blinded, and three independent fields of view counted per section, with 3 sections in total. At 24 hours there is a greater number of MHC class II cells were counted, this is possibly due to the mechanical damage of the injection, resulting in an increased recruitment of DCs ($p=0.30$). At 36 hours, CCL20 encoded plasmid and the empty vector were equivalent ($p=0.13$). The lack of significant difference at 36 hours between control and dosed skin is likely to be due to the lag in recombinant CCL20 production and the time required for the recruitment of significant DC numbers. An increase can be observed between control and CCL20 encoded plasmid dosed skin at 48 hours, suggesting that the transgene is being expressed and MHC class II cells are being recruited ($p=0.01$). There was no significant difference in recruitment at 72 hours between the CCL20 encoding plasmid and the empty vector plasmid ($p=0.53$). The recruitment of DCs to the skin by CCL20 is well

documented (Charbonnier *et al.* 1999; Dieu-Nosjean *et al.* 2000; Nakayama *et al.* 2001), and is further evidence to support the biological activity of our plasmid encoded CCL20.

The lack of significant difference between control and recombinant plasmid encoded CCL20 dosed skin at the 72-hour time point could be explained by a number of factors. It is possible that there was a CpG mediated immune response against plasmid transfected cells, and therefore a dramatic fall in the local levels of recombinant CCL20 due to their elimination. This would allow any DCs present to migrate to draining lymph nodes by their default migratory pathways (CCR7 and CCL21). Alternatively, the DCs at this point may have reached a stage of maturity when they naturally down regulate CCR6, concomitantly up-regulating CCR7 inducing migration to draining lymph nodes. However, we expect the initial priming events to have occurred before 72 hours and migration from the site of immunisation to the draining lymph node is required to prime naïve T cells. Therefore, these observations suggest that increased DC number migrate to the draining lymph node and may lead to heightened immune response.

With evidence that the CCL20 expressed by the plasmid elicited biological activity *in vivo*, a study was undertaken to examine the efficiency of CCL20 in enhancing the response to the target antigen secreted alkaline phosphatase (SEAP). After the secondary boost, both humoral and cellular responses were measured. In the experimental groups using plasmid encoding SEAP either with or without CCL20, there was no detectable antibodies production or T-cell proliferation. However, in the positive control, of

recombinant SEAP administered with the adjuvant cholera toxin, both antibodies and T-cell proliferation could be detected. The plasmid conformed to the initial requirements of having a purity of 1.8 as determined by the absorbance at 260nm versus 280nm, and it also transfected the cells at equivalent commercially derived plasmid preparations. However, when the plasmid was re-examined after the course of immunisations, by gel electrophoresis, the plasmid was degraded. *In vitro* transfections resulted in levels SEAP production 2 logs lower than with a new preparation of SEAP vector. This suggests there was a problem within the plasmid preparation, and that if time permitted a future attempt would give a definitive answer as to the effect of CCL20.

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A possible explanation for the absence of an adjuvant effect observed by CCL20 in this study is the recent report that CCL20 may play a role in retaining CCR6 expressing cells in the tissue that have been previously recruited by another chemokine MCP-4(macrophage chemotactic protein-4) of CCR2 positive cells (Vanbervliet *et al.* 2002). This gives rise to the potential of two opposing hypothesis, is there a threshold effect, where upon above a certain level of CCL20, CCR6 positive cells are recruited. Or is there an additive effect, where upon the greater the concentration of CCL20 the more CCR6 positive cells are recruited. If the first hypothesis is true, then the use of CCL20 as a genetic adjuvant would be limited once the threshold has been reached, however, if the second hypothesis was true, then the adjuvanticity of CCL20 could conceivably be very powerful, and dependant upon the transfection efficiency of the plasmid.

Here we have produced biologically active recombinant CCL20 and shown that plasmid encoded CCL20 is expressed at the site of administration. This resulted in increased DC influx. However, it was not possible to show an enhanced immune response as a result of increased local DC number, which may have been due to problems with the immunogen encoding vector as discussed. It is likely if these experiments were repeated a significant difference would be found.

With both recombinant murine CCL20 and monoclonal antibodies to CCR6 now commercially available, investigation of murine chemotactic responses would be much easier. Investigating if CCL20 retains cells or induces active migration would be important to ascertain. Examining the adjuvanticity to a plasmid encoded antigen is essential, however, possibly the co-immunisation with a MCP-4 encoded plasmid might induce a broader spectrum response.

Chapter 6 – Discussion and Future Studies

6.1 Discussion

Plasmid DNA expression vectors have many attributes that make it an ideal candidate as a vaccine vector. Plasmid DNA is easy to manufacture and manipulate, and has been shown to induce not only cellular, but also humoral immunity. Many routes of immunisation have been investigated to deliver plasmid DNA, including intramuscular (Danko and Wolff 1994; Yang *et al.* 1995), intravenous (Wu and Wu 1988; Kawabata *et al.* 1995), subcutaneous (McCluskie *et al.* 1999), intranasal (Wheeler *et al.* 1996; Gao *et al.* 1997) and intradermal (Rakhmilevich *et al.* 1996; Choi *et al.* 1997; Degano *et al.* 1998) to the less patient compliant route of targeting plasmid DNA to lymph nodes (Lehner *et al.* 1996; Lu *et al.* 1998). The main disadvantages of plasmid DNA vaccines are the amount of DNA necessary to elicit protective immune responses in man, and the degradation of the DNA depending upon the route of immunisation.

The hypothesis of this thesis is that the by increasing the delivery of plasmid DNA both to mitotic and non-mitotic cells, or by increasing the number of APCs presenting the target antigen, the immunogenicity of plasmid DNA vaccines can be increased. Chapter 3 discussed the application of cationic lipids in facilitating and improving binding and uptake of plasmid DNA into cells and protection of plasmid DNA from degradation. Chapter 4 examined a novel peptide that could act in a similar manner to cationic lipids, but could cross the cell membrane via a non-endocytic mechanism, and chapter 5 discussed the role of a new potential molecular adjuvant, to increase the efficacy of the immune response, and thus potentially reduce the amount of plasmid DNA required.

Within plasmid DNA vaccine development, the route of immunisation is an essential consideration. An ideal goal for future vaccine administration would include a needle free, inexpensive method of vaccine delivery targeting a mucosal surface, the primary site of transmission for many pathogens. The mucosal surfaces include the nasopharynx, vagina, rectum, and gastrointestinal tract. In each of these environments, degradation of naked plasmid DNA has been reported after a few minutes, with a significant reduction in transgene expression (Nomura *et al.* 1997). Delivery of plasmid DNA by the intranasal route has been shown to induce both a mucosal and a systemic response (Kuklin *et al.* 1997; Asakura *et al.* 1999; Klavinskis *et al.* 1999), with the generation of both Th_1 and Th_2 responses (Kanellos *et al.* 2000; Debin *et al.* 2002; Tanaka *et al.* 2002), which makes this an optimal route for mucosal immunisation.

There are several methods of protecting plasmid DNA from endonuclease degradation for optimal efficiency of gene expression. We have shown that complexing plasmid DNA with cationic lipids, can be an effective way of increasing the efficiency of transfection. However, the cationic lipids are preferentially targeted to the endosome and require aid in escaping to the cytosol (Nakanishi and Noguchi 2001). The majority of plasmid DNA can be found in the endosome after cationic lipid mediated transfection. There is a constant loss of plasmid DNA at each cellular barrier, including cell entry, escape from the endosome and nuclear entry (Zuhorn and Hoekstra 2002). Identifying non-viral mechanisms that overcome these cellular barriers is of critical importance. The chemical composition of the cytofectins and the formulation of the plasmid DNA with the cationic lipid can attribute to the variances observed in the transfection efficiency (Thierry *et al.*

1997; Ferrari *et al.* 2002). We have examined a range of variables, examining the relative amount of lipid complexed with plasmid DNA to determine the most efficient ratio for transfection, and rationally designing a protocol for intranasal immunisation.

In NALT, DCs are interspersed among the epithelial lining of the nasopharynx. We have shown that following transfection with a GFP expressing plasmid that the transgene can be observed in sections of NALT tissue, and that the plasmid DNA is preferentially taken up by the mucososecretory glands. When cells from NALT and its draining lymph nodes, the CLN, are purified by flow cytometry into DC's and T and B cells, the transgene was observed within the DCs but not within the lymphoid subsets. DCs are a prime target for any potential vaccine candidate, as direct transfection of DCs will result in transgene expression and antigen displayed on both MHC class I and class II, broadening the depth of the response. It is still currently unclear whether plasmid DNA directly transfects DCs, or whether DCs are cross-primed from other cells, for example epithelial cells, that contain the transgene. In support of our study, antigen and reporter genes have been demonstrated in macrophages, Langerhan' cells and DCs present both at the site of DNA vaccine delivery and in draining lymph nodes (Chattergoon *et al.* 1998; Akbari *et al.* 1999; Barnfield *et al.* 2000). This suggests that APCs are transfected *in situ* and migrate to draining lymph nodes or alternatively are transfected within lymph nodes following lymphatic drainage of plasmid DNA (Barnfield *et al.* 2000). Conclusively, data has demonstrated plasmid encoded mRNA in isolated DCs following either intradermal or mucosal vaccine delivery (Bouloc *et al.* 1999; Barnfield *et al.* 2000). Interestingly, direct transfection of DCs has been extremely difficult to demonstrate with plasmid DNA *in*

vitro (Denis-Mize *et al.* 2000), suggesting instead that *in vivo* the transgene is present due to cross-priming. However, it should be noted that bone marrow derived DCs, although displaying many characteristics similar to DCs within the mucosa, are generated in the presence of cytokines that are rather not present, or at different concentrations, than those expressed *in vivo*. Studying DCs *ex vivo* is also problematic since physical removal of DCs from connective tissue can also alter their properties, and induce maturation. Thus although *in vitro* manipulation of DCs is a powerful tool, it cannot replicate an *in vivo* environment. It is well established that DCs phagocytose apoptotic cells (Albert *et al.* 1998; Rovere *et al.* 1998; Van Zanten *et al.* 2002), thus considering that cationic lipids have been reported to be toxic (Dass 2002), it is possible that transfected cells, undergo apoptosis and expressed plasmid DNA encoded protein is taken up by the DC.

DCs are highly phagocytic, and all phagocytosed material is rapidly degraded within the endosome. Thus a novel delivery system that can bypass the endosome has great potential for transfecting DCs. Peptides from the proteins Antennapedia, HIV-Tat and VP20 from HSV have all been reported to translocate across the cellular membrane in an endosome independent manner (Morris *et al.* 2001). Primarily these peptides have been investigated for their potential to co-translocate a fused peptide, for efficient delivery into the cytosol. We have shown that a peptide containing the translocation domain of the Antennapedia peptide, fused to a peptide consisting of 16 lysines via a 3 glycine hinge, can transport DNA across the membrane, and expression of the plasmid encoded transgene can be observed within cell lines. However, the transfection efficiency could not be replicated when a new batch of peptide from the same manufacturer was used. This could be due to

a number of reasons, storage of the peptide is critical, trace contaminations within the peptide can affect transfection, formulation of the peptide (pH or salinity) is critical.

One of the main disadvantages of plasmid DNA is the necessity for large doses of DNA to facilitate the generation of an immune response in man. There have been many studies studying the effects of co-immunising chemokines, cytokines and growth factors with a plasmid encoded target antigen in the attempt to reduce the quantity of plasmid DNA required to induce a response (Okada *et al.* 1997; Xin *et al.* 1999; Ou-Yang *et al.* 2002; Luo *et al.* 2003). The most successful strategies within the literature involve the recruitment of DCs to the site of immunisation, and an increase in the amount of inflammation induced by the plasmid (Lu *et al.* 1999). The identification of CCL20, a chemokine that was reported to specifically attract immature DCs, suggested this chemokines as a prime candidate to enhance the immunogenicity of mucosally administered plasmid DNA vaccines (Hieshima *et al.* 1997). We cloned CCL20 into an expression vector and studied the biological activities of this chemokine on a variety of cell lines and primary cells. Preliminary data from this thesis suggested that CCL20 does not induce migration in a transmigration assay, or stimulate a calcium flux in the cells tested. Within the literature, there is a divergence between humans and mice in the recruitment of CCR6 expressing cells (especially $CD34^+$ haemopoetic precursor cells cultured in GM-CSF) to CCL20. Fushimi et al, 2000, reported that supernatants of cells infected with a vaccinia virus (VV) encoding CCL20 were chemotactic for bone marrow derived DCs. They also reported that intratumoral immunisation with VV encoding CCL20 induced an accumulation of DCs *in vivo*. Biragyn et al, 2001 also reported the

role of CCL20 and the β-defensins in binding to CCR6 and attracting bone marrow derived DCs. Iwasaki and Kelsall, 2000, isolated DCs from within the subepithelial dome of the Peyer's patches and showed transmigration of these cells in response to CCL20. However, in another study, transmigration of DCs to murine CCL20 could not be observed (Ogata *et al.* 1999). We have studied the transmigration of murine bone marrow derived DCs with both commercially produced recombinant murine CCL20 and supernatants cell lines transfected with an expression plasmid encoding murine CCL20, without any migration being observed. There are reports that depending upon the cytokine environment within the bone marrow derived culture, different phenotypes and characteristics of the resulting DCs can be generated. Analysis of the phenotype of the DCs, for the cultures generated in this study, indicated expression of CCR6, the receptor for CCL20. It is possible that murine CCL20 does not induce migration since Iwasaki and Kelsall, 2000, Birygan *et al*, 2001 and Fushimi *et al*, 2000, used human CCL20 in their studies to demonstrate recruitment. The data for this thesis complement the observation of Ogata et al, 19999, which were unable to detect significant migration of murine DC by murine CCL20.

It has recently been proposed that CCL20 works in combination with CCL13 to induce transmigration *in vivo*. A sequential triggering of chemokine receptors has been reported to induce migration of DCs from the blood to the site of infection. Vanbervliet et al, 2002, suggest that $CD14^+$ precursor monocytes initially migrate to CCL13, a CCR2 binding chemokine, and then migrate along a gradient to CCL20. Both chemokines have been observed to produce complimentary gradients, with CCL13 expressed in basal

epithelial cells at the contact of blood vessels, while CCL20 expression is restricted to epithelial cells bordering the external milieu. The migration of blood monocytes through the tissue in response to CCL13 may mature and upregulate surface expression of CCR6 and thus induce responsiveness to a CCL20 gradient.

This thesis demonstrated that expression of plasmid DNA encoded proteins could be enhanced by incorporation of cationic lipids or novel peptides. It has also demonstrated that a plasmid encoding CCL20 induces recruitment of DCs *in vivo*, thus potentially enhancing the immune response when co-administrated with a plasmid encoding an antigen. These modifications to vaccine design may incrementally facilitate the translation of this vaccine technology to the clinic.

The present study undertook to enhance the efficiency of plasmid DNA delivery *in vivo* by complexing the DNA with cytofectins or with peptides. A further aim was to enhance the immunogenicity of plasmid DNA by recruiting DCs at the site of immunisation. We have shown that optimisation of the molar ratio of plasmid DNA to cytofectins can enhance the production of the transgene both *in vitro* and *in vivo*, and that the transgene can be expressed in DCs at the site of inoculation and at the draining lymph node. We have also shown that transfection can be achieved using an Antennapedia peptide delivery system, however further investigation is necessary to elucidate if this peptide facilitates plasmid DNA uptake in an endosomal independent manner. Finally we have shown that the chemokine CCL20 can attract DCs *in vivo*, and although the adjuvant effect of this chemokine in augmenting immunogenicity of an antigen encoded plasmid was undertaken, the results were inconclusive, and need to be reassessed.

A recent observation suggested that blood DCs are recruited through a sequential activation of the chemokines receptors CCR2 and CCR6 (Vanbervliet *et al.* 2002). This suggests that administration of a plasmid that encodes CCL13 and CCL20 using an internal ribosome entry site (IRES) could increase the recruitment of DCs to the site of immunisation. Ligation of CCR2 by CCL13 has been shown to induce migration of DCs (de la Rosa *et al.* 2003; Liu *et al.* 2003). Although CCL13 has not been used as a molecular adjuvant, there is evidence to indicate that another CCR2 ligand, CCL2 enhanced the immunogenicity of a DNA vaccine (Eo *et al.* 2001). Co-immunisation with plasmid DNA encoding gB of herpes simplex virus and CCL2 enhanced the immune response to the target antigen, which was skewed to a Th2 response inducted by the immunoglobulin isotypes and IL-4 expression produced by CD4 T cells (Eo *et al.* 2001). If complementary gradients of CCL13 and CCL20 are established *in vivo* as reported by Vanbervliet et al, 2001 then an increase in the recruitment of DCs should be achieved by the dual plasmid encoded expression of both CCL13 and CCL20.

Chemokines expressed as a fusion protein with an antigen may have the potential to improve the immunogenicity of the cognate antigen. Immunisation with plasmid DNA encoding a fusion protein of and a target antigen has been reported to not only facilitate the uptake of the fusion protein to DCs via CCR6, but also to induce migration of immature DCs (Biragyn *et al.* 2001). Using the same rationale, plasmid DNA encoding the 16 amino acid Antennapedia peptide in tandem with a target antigen could be constructed. Recently a fusion protein containing the ovalbumin SIINFEKL CTL peptide

was shown to induce a strong CTL response (Pietersz *et al.* 2001). This suggests that CTL epitopes can be transfected directly into the cytosol and gain access to newly synthesised MHC class I molecules. Based on this premise, direct transfection of DC could be bypassed by transfecting stromal cells with plasmid DNA encoding a secreted fusion protein encoding Antennapedia and the target antigen for delivery to the DC.

Recent studies have indicated that expansion of DC subsets administration of growth factors, for example GM-CSF or *fms*-like tyrosine kinase ligand (Flt3L), can increase the immunogenicity of a co-administered target antigen. Flt3L, a growth factor for early haematopoietic progenitor cells (Lyman *et al.* 1993) has been reported to induce the expansion of DCs (Maraskovsky *et al.* 1996). and generate a strongly polarise Th1 response (Morelli *et al.* 2000). Co-administration of plasmid DNA encoding HBV core antigen and Flt3L showed that the Flt3L suppressed humoral responses to the target antigen (Kwon and Park 2002). Another study showed that inoculation with plasmid DNA encoding Flt3L, increased the number of DCs at both the site of immunisation and the draining lymph node (Sang *et al.* 2003).

CD40L, a member of the TNF gene family that is expressed on T cells upon Ag recognition may represent another genetic adjuvant. CD40L is upregulated following TCR ligation and induces co-stimulatory activity on DCs via engagement of CD40, which provides a second co-stimulatory signal for full activation of T cells. (Grewal and Flavell 1998). Plasmid DNA encoding CD40 DNA was found to enhance cellular immune responses such as induction of IFN-γ and CTL activity when mice were

vaccinated with plasmid DNA encoding β-galactosidase (Gurunathan *et al.* 1998). Furthermore, co-immunisation with plasmid DNA encoding CD40L with the F and G proteins from Rous Sarcoma Virus (RSV) has been reported to enhance Th1 (IL-2 and IFN-γ) cytokine responses to the co-inoculated antigens, increase the expression of TNF-α and Nitric Oxide, increase the humoral response to the target antigens and accelerate virus clearance (Tripp *et al.* 2000).

In conclusion, plasmid DNA has a great potential as a vaccine vector, with its ease of manufacture and manipulation. Continual advances in elucidating the mechanisms of regulating the immune response, is revealing new and novel molecular adjuvants. Using chemokines, growth factors or co-stimulatory molecules, immune responses can be augmented and the amount of plasmid DNA required to induce protection against infection disease in man may be reduced.

6.2 Future Studies

The aims of the strategies discussed within this thesis focus on improving the immune response generated towards a target antigen, with the ultimate aim of improving the efficacy of plasmid DNA vaccination. Examining the CTL and antibody responses generated by the vaccine alone or combined with CCL20, Antennapedia or lipid formulation can identify the immunogenicity of the strategy. However, the true test of the effectiveness of these candidates listed within is to apply these approaches to prevent disease or viral clearance upon pathogen challenge. The principle behind the approach was for a simple, effective mucosal vaccine that can be given via the intranasal route, thus negating the necessity for a needle based delivery system. Therefore, a model system that is clearly defined and targets the mucosa would be appropriate for showing the efficacy of these approaches. One such challenge system is the use of influenza virus.

Influenza is normally cleared from the lungs within 10 days post infection, with viral RNA sequences undetectable in the lung by day 14, within the O-NALT within 12 days, and within the D-NALT within 17 days (Liang *et al.* 2001). By examining antibody titres, CTL responses, and viral clearance rates, one can ascertain the increased effectiveness of these modified vaccine strategies, and that by their addition one can reduce the amount of plasmid DNA required to induce an immune response.

Another mucosal viral challenge system is by Herpes Simplex Virus. One of the key attributes of a mucosal vaccine is the induction of mucosal responses at distal sites.

Immune responses generated by plasmid DNA encoding HSV glycoprotein B delivered intranasally have been observed to include interferon-producing T and NK cells and the IgG2a isotype immunoglobulin. With co-immunisation of cytokine encoded DNA (IL-12 or IL-18) have shown enhanced resistance to lethal vaginal HSV infection, however the response was never as high as that observed with UV-inactivated HSV . By using the methods investigated within the thesis, the immune response towards the plasmid DNA can be increased.

Appendices

Appendix 1

COMMONLY USED BUFFERS AND PROCEDURES

All chemicals were of analytical grade and all solutions were made in glass with double distilled water.

1.1 STANDARD BUFFERS AND REAGENTS

(1). Phosphate Buffered Saline (pH 7.4):

Sodium Chloride	100mM
Potassium Chloride	2.7mM
Na_2HPO_4	80mM
NaH_2PO_4	20mM
pH to 7.4	

(2). Tris Buffered Saline (pH 7.0):

Tris base	20mM
Sodium Chloride	137mM
PH to 7.0	

(3). EDTA (0.5M pH 8.0):

Disodium EDTA	0.5M

(4). STE:

NaCl	0.1M
Tris Cl (pH 8.0)	10mM
EDTA (pH 8.0)	1mM

(5). TE:

Tris Cl (pH 8.0)	10mM
EDTA (pH 8.0)	1mM

(6). Plasmid DNA Maxi Preparation Solutions

Solution 1

Glucose	50mM
Tris-Cl (pH 8.0)	25mM
EDTA (pH 8.0)	10mM
Lysozyme	1mg/ml

Solution 2

Sodium Hydroxide	0.2M
SDS	1%

Solution 3

potassium acetate 5M	60 ml
glacial acetic acid	11.5 ml

ddH2O	28.5 ml

Total Volume: 100 ml

Filter sterilize. The resulting solution: 3 M potassium, 5 M acetate and is pH 4.8.

(7). TBE Buffer:

Tris borate	0.089M
Boric acid	0.089M
EDTA (pH 8.0)	0.002M

(8). TAE Buffer:

For 1 litre of 50x stock solution:

Tris base	242g
Glacial acetic acid	57.1 ml
EDTA (pH 8.0) 0.5M	100ml

Final working solution: Tris acetate 0.04M, EDTA 0.001M

(9). SDS Acrylamide Gel

(A). 10% Separating Gel:

40% Acrylogel (BDH, Poole, U.K)	2.5 ml
1.5M Tris pH 8.8	2.5 ml
10% SDS	0.1 ml
Water	4.83 ml

10% APS	60 µl
TEMED	7 µl
Final volume 10 ml	

(B). Stacking Gel:

40% Acrylogel (BDH, Poole, U.K)	0.5ml
0.5M Tris pH 6.8	1.25 ml
10% SDS	50 µl
Water	3.5 ml
10% APS	35 µl
TEMED	4 µl
Final volume 5 ml	

(10). Reducing Sample Buffer for SDS Page Analysis:

Tris Cl pH 6.8	0.125M
10% SDS	0.14M
Glycerol	20% v/v
2-mercaptoethanol	2% v/v
Bromophenol Blue	0.2mM
Deionised water	0.03M

(11). 5x Electrode (Running) Buffer:

Tris	15g

Glycerine	72g
SDS	5g

Dissolved in 1 litre distilled water. Stored at 40C; 300 ml

5x stock diluted with 1.2 litre distilled water.

(12). Western Blot Transfer Buffer

Glycine	0.192 M
TRIS base	25 mM
Methanol	20%

(13). Terrific Broth:

Bactotrytone	12g
Bacto-yeast extract	24g
Glycerol	4 ml

Make in 900 ml and after autoclaving add 100 ml of 0.17M KH_2PO_4, 0.72M K_2HPO_4.

(14). Hanks Balanced Salts (pH 7.3)

$CaCl_2$	2mM
NaCl	145mM
KCl	5mM
$MgCl_2$	1mM
D-glucose	5mM

Hepes	20mM

(15). RPMI^{+} or DMEM^{+}

Penecillin	100U/ml
Streptomycin	100µg/ml
L-Glutamine	2mM
Fetal Calf Serum (Heat Inactivated)	10%

In RPMI-1640 or DMEM.

(16). Heamatoxylin Counter Stain

1 litre of solutions A and B are mixed with 20ml solution C.

Solution A:-

10% aluminium ammonium sulphate solution

Solution B:-

10% Chloral Hydrate

0.5% Citric Acid

0.1% Sodium iodate

Solution C:-

10% Haematoxylin in 100% Ethanol

1.2 MEASUREMENT OF pH

pH was measured using PHM standard pH meter (Radiometer) with a combination gel epoxy electrode (Whatman,). Measurement was accurate to ± 0.05 pH units.

1.3. AUTOCLAVING

All buffers solutions used in cell studies were autoclaved at 15 pounds per square (psi) inch for 30 minutes / 500 ml in an Express RG2 portable autoclave (Arnold and Sons, U.K).

Plastic tips for micropipettes for culture studies were autoclaved at 15 psi for 15 minutes in an Autoclave-SUA (British Steriliser Co, U.K).

Appendix 2

SOURCES OF REAGENTS

2.1. CHEMICALS

Acetic acid – BDH, Poole, U.K.

Bovine Serum Albumin – Sigma, Poole, U.K.

Bromophenol blue – BDH, Poole, U.K.

Commassie brilliant blue – Sigma, Poole, U.K.

Disodium dihydrogen orthophosphate – BDH, Poole, U.K.

EDTA (ethylenediaminetetracetic acid) – BDH, Poole, U.K.

Ethanol (absolute) – BDH, Poole, U.K

Ethidium Bromide – BDH, Poole, U.K.

Glucose – BDH, Poole, U.K.

Glycerol - BDH, Poole, U.K.

Glycine – BDH, Poole, U.K.

Glutaralderhyde – BDH, Poole, U.K.

Hydrochloric acid (concentrated) – BDH, Poole, U.K.

Hydrogen Peroxide (30% w/v) – BDH, Poole, U.K.

LiCl (lithium chloride) – BDH, Poole, U.K.

Low EEQ, low melting point (LMP) agarose – Sigma, Poole, U.K.

Methanol – BDH, Poole, U.K.

2-β-mercaptoethanol (2-ME) – BDH, Poole, U.K.

N-N-Dimethlyformamide – Sigma, Poole, U.K.

Nonident P40 (NP40) – BDH, Poole, U.K.

O-phenylenediamine Dihydrochloride – Sigma, Poole, U.K

Paraformalderhyde – Sigma, Poole, U.K.

Potassium acetate – BDH, Poole, U.K.

Sodium azide – BDH, Poole, U.K.

Sodium chloride – BDH, Poole, U.K.

Sodium dihydrogen orthophosphate – BDH, Poole, U.K.

Sodium dodecyl sulphate (SDS) – BDH, Poole, U.K.

Sodium hydroxide – BDH, Poole, U.K.

Tris (hydroxymethyl) methylamine (Tris) – BDH, Poole, U.K,

Triton X-100 – BDH, Poole, U.K.

Tween-20 – BDH, Poole, U.K.

2.2. ENZYMES, ENZYME INHIBITORS (INCLUDING PROTEINS), MITOGENS

Brefeldin-A – Sigma, Poole, U.K.

Deoxyribonuclease-I – Sigma, Poole, U.K.

Levaimisole – Sigma, Poole, U.K.

Lysozyme – Sigma, Poole, U.K.

Monensin – Sigma, Poole, U.K.

2.3. CULTURE PRODUCTS

DMEM – Gibco BRL, Paisley, U.K.

Fetal Calf Serum – PAA Laboratories, Cambridge, U.K.

L-Glutamine - Gibco BRL, Paisley, U.K.

RPMI-1640 – Sigma, Poole, U.K.

Penicllin - Gibco BRL, Paisley, U.K.

Streptomycin - Gibco BRL, Paisley, U.K.

2.4. CHEMOKINES

CCL20 – Peprotech, London, U.K.

CCL20 – R&D Systems, Abingdon, U.K.

GM-CSF – Peprotech, London, U.K.

GM-CSF – R&D Systems, Abingdon, U.K.

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